Studies on probiotics for the control of vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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Abstract

Kocuria SM1 and *Rhodococcus* SM2 were administered to juvenile rainbow trout as dietary supplements dosed at ~10^7–10^8 cells g^{-1} of feed for two weeks, and conferred protection against *Vibrio* infections. Thus, use of SM1 and SM2 led to a significant ($P < 0.05$) reduction in mortalities, i.e. 12–15% (relative percent survival, RPS = 81–85%) and 15–20% (RPS = 73–80%) after challenge with *V. anguillarum* and *V. ordalii*, respectively, compared to the 80% and 74% mortalities among the respective controls. A two-week feeding regime, compared with 1–4 weeks, for SM1 led to the maximum reduction in mortalities after challenge with *V. anguillarum*. The use of an equi-mixture of SM1 and SM2 led to significantly enhanced survival against vibriosis, but the result was not better than the use of single cultures. Moreover, use of SM1 for two weeks led to protection for up to 4 weeks. Fish inoculated with cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2 demonstrated better protection against challenge with *V. anguillarum*, although extracellular proteins (ECPs) fared less well. The mode of action reflected competitive exclusion (= antibiosis), nutrition (= positive effects on growth), and stimulation of cellular and humoral innate immunity, notably greater head kidney macrophage phagocytic, respiratory burst, peroxidase and bacterial killing activities, and elevation of leucocytes, globulin, protein, complement and lysozyme levels. These results demonstrate the efficacy of dietary bacteria as probiotics for the control of vibriosis in rainbow trout.

To my son Zuhayr

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Declaration

I, S.M. Sharifuzzaman, hereby declare that I am the author of this thesis. All the work described in this thesis in my own, except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of references.

S.M. Sharifuzzaman

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List of abbreviations and symbols

%	percent
~	approximately
<	less than
>	greater than
±	plus or minus, error margin
×	multiply/times
≤	less than or equal to
≥	greater than or equal to
°C	degree centigrade
Ab	absorbance
BC	before Christ
BLAST	basic local alignment search tool
bp	base pair
cfu	colony forming unit
cm	centimeter
DNA	deoxyribonucleic acid
e.g.	for example
g	gram
g	gravitational force
h	hour
i.e.	'id est': that is
i.m.	intramuscularly
i.p.	intraperitoneally
Ig	immunoglobulin
kDa	kiloDalton
kpb	kilo base pair
L	litre
LD_{50}	lethal dose to kill 50% of the population
M	mol
mg	milligram
min	minute
mL	millilitre
mM	millimolar

nm	nanometer (= unit for wavelength)
OD	optical density
PCR	polymerase chain reaction
pmol	picomole
RPS	relative percent survival
spp.	species
V	volt
v/v	volume to volume ratio
w/v	weight to volume ratio
w/w	weight to weight ratio
µg	microgram
µL	microlitre
µm	micrometer

List of publications

Sharifuzzaman SM, Austin B (2009) Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. Fish & Shellfish Immunology 27: 440–445.

Sharifuzzaman SM, Austin B (2010a) *Kocuria* SM1 controls vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Journal of Applied Microbiology 108: 2162–2170.

Sharifuzzaman SM, Austin B (2010b). Development of protection in rainbow trout (*Oncorhynchus mykiss*, Walbaum) to *Vibrio anguillarum* following use of the probiotic *Kocuria* SM1. Fish & Shellfish Immunology 29: 212–216.

Abbass A, Sharifuzzaman SM, Austin B (2010) Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 33: 31–37.

CHAPTER 1 – INTRODUCTION

1.1 General introduction

Aquaculture (= the production of aquatic plants and animals in controlled conditions), which has been commercially practiced in China since 500 BC (see Pillay and Kutty 2005), continues to increase globally and accounts for about half (47%) of all food fish supplied in 2006 (Figure 1.1). It is viewed increasingly to have the greatest potential to meet the growing demand for aquatic food stimulated by the static or depleting nature of capture fisheries and the concomitant market forces of increased demand (FAO 2009). For example, production of rainbow trout (*Oncorhynchus mykiss*) in Scotland stood at 7,414 tonnes in 2007 from a production of 4,263 tonnes in 1994 (FRS 2008). Scottish aquaculture is estimated to have had a farm gate value of £346 million in 2007, which includes £324 million for farmed Atlantic salmon, approximately £14 million for rainbow trout, and around £5 million for shellfish. The industry also makes a significant contribution to the Scottish rural economy, especially among the communities of the western and northern isles, where it directly generated ~1,500 jobs and through linked activities a further 4,700 indirect employment such as through processing and packaging activities (source: The Scottish Government; http://www.scotland.gov.uk).

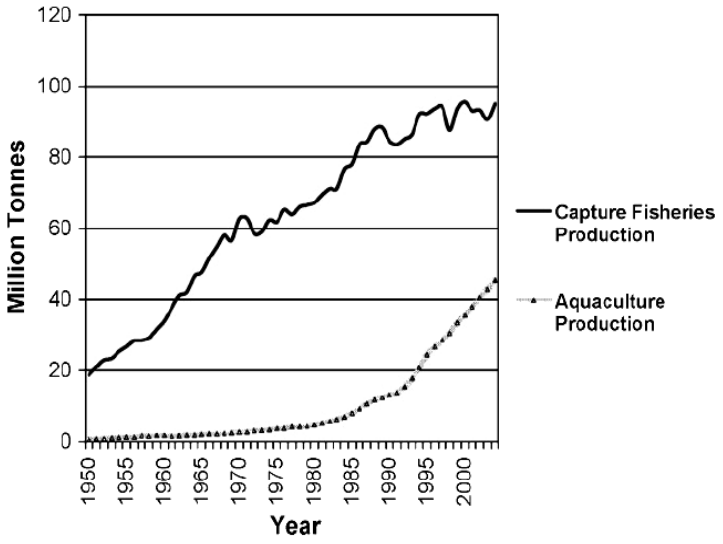

Figure 1.1 World fish production (source: Jiang 2009).

Farming of rainbow trout was introduced to the U.K. in the 1950's by a Danish entrepreneur, and since that time the activity has shown a steady increase in total production and has become an important industry, particularly in England. There are almost 360 trout farms in the U.K. producing ~16,000 tonnes per annum. Figure 1.2 shows the active rainbow trout farms in Scotland. Among the trout species, rainbow

trout is the most popular one to farm due to its better tolerance to warm water, faster growth, larger size and fitness with farming systems (source: British Trout Association; http://www.britishtrout.co.uk). Other popular species include brown trout/sea trout (*Salmo trutta*), golden trout (*Oncorhynchus mykiss aguabonita*) and blue trout, though in smaller amounts (Figure 1.3). Most of the trout is farmed in freshwater, usually using tanks, ponds, cages or raceways, with a small quantity farmed in sea cages.

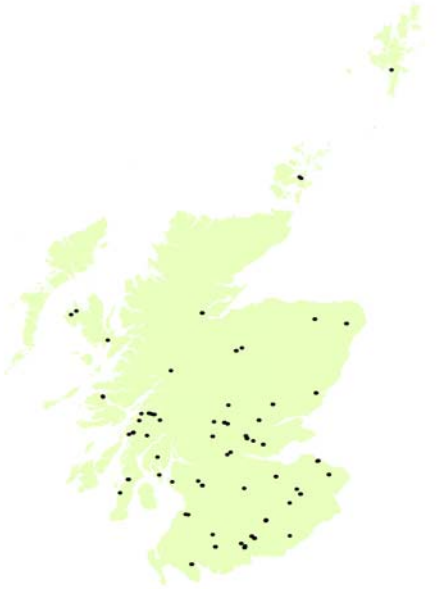

Figure 1.2 Active rainbow trout sites (black dots on map) in Scotland, 2007 (source: FRS 2008).

Figure 1.3 Various trout species farmed in Scotland: (A) rainbow trout (highly-spotted tail/fin often with a wide band of red, pink or mauve along the side/flank), (B) brown trout (highly-spotted skin, with brown background colouration varying to yellow, and with black, orange, or red spots) and (C) blue trout (bright silver underside and striking blue topside). With acknowledgements to http://www.coastalthings.com; http://www.bewl.co.uk; http://www.timberwolfoutfitters.com.

Largely two types of farming systems are employed in aquaculture, either open or closed systems (Tacon and Forster 2003). The open system, also known as semi-culture, is the easiest and least technology demanding way to grow fish where farming takes place under relatively natural conditions with little manipulation, and the stocking

densities do not exceed those found in nature, for example, mollusks grown on racks or hanging rafts in favourable sites. Closed systems employ intensive management of a production system where the farming is optimized by totally controlling the organisms and their environment, thus ensuring maximal growth. This type of aquaculture is commonly regarded as intensive aquaculture, for example involving recirculating systems (Castro and Huber 1997). The stocking densities of such systems greatly exceeds those of open systems, i.e. more weight (Kg) of fish than the total volume (L) of water, consequently the yields are very high. However, the overall cost and management increases, as does the potential for catastrophic loss.

Table 1.1 Some major pathogens of farmed fish and shellfish species (after Owens 2003; Leong 2008).

Group	Genera, etc.
Viruses	DNA genomes: families include *Iridoviridae*, *Adenoviridae*, *Herpesviridae*
	RNA genomes: families include *Picornaviridae*, *Birnaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Caliciviridae*, *Togaviridae*, *Nodaviridae*, *Nidovirales*, *Retroviridae*, *Coronaviridae*
Bacteria	Rickettsiales, *Aeromonas*, *Enterococcus*, *Flavobacterium*, *Flexibacter*, *Pseudoalteromonas*, *Pseudomonas*, *Streptococcus*, *Vibrio*
Fungi	*Aphanomyces*, *Branchiomyces, Lagenidium*, *Saprolegnia*, *Sirolipidium*
Protozoa	Amoebae: *Neoparamoeba* Flagellates: *Hexamita*, *Ichthyobodo* Ciliates: *Ichthyophthirius*, *Trichodina* Sporozoans: *Bonamia*, *Loma*, *Marteilia*, *Perkinsus*
Helminths	*Dactylogyrus*
Nematodes	*Eustrongylides*
Annelids	*Polydora*
Crustaceans	Fish ‘lice’: Isopods Fish ‘lice’: Branchiura: *Argulus* Copepods: *Lernaea*, *Ergasilus*, *Mytilicola* Crabs: Pinnotherids
Gastropods	Pyramidellids

Although aquaculture is regarded to be the fastest growing animal food-production system in the world, disease outbreaks are often the key limitation to the expansion of

this sector in many parts of the world (Subasinghe 2005). In fact, all forms of aquaculture are susceptible to outbreaks of diseases as many pathogens are normal inhabitants of the aquatic environment (Pillay 2004). Taxonomically, the pathogens are ranged from single stranded RNA viruses, e.g. infectious salmon anaemia (ISA) to complex parasitic crustaceans, e.g. sea lice (Murray and Peeler 2005), and broadly classified as viruses, bacteria, fungi, parasites, protozoa and metazoans – Table 1.1 (Owens 2003; Austin 2005; Bondad-Reantaso *et al.* 2005; Austin and Austin 2007). Particularly, the problems of *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus* biotype 2 and *V. harveyi* with several important aquaculture species, such as salmonids, turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), striped bass (*Morone saxatilis*), European eel (*Anguilla anguilla*), ayu (*Plecoglossus altivelis*), cod (*Gadus morhua*), sea bream (*Sparus aurata*), and shrimp may lead to huge economic loss (Karunasagar *et al.* 2005; Toranzo *et al.* 2005; Austin and Austin 2007). Certainly, the epidemiology of bacterial diseases in Norway between 1991 and 2000 detected vibriosis as a major obstacle to the future growth of rainbow trout, halibut (*Hippoglossus hippoglossus*), turbot, cod and European eel farming (Lillehaug *et al.* 2003). Consequently, control of fish diseases is of utmost importance. This thesis addresses control strategy of vibriosis in rainbow trout caused by infections due to *V. anguillarum* and *V. ordalii* through application of probiotics as a modern approach to disease control.

1.2 Diseases of farmed fish

The term ‘disease’ denotes a certain negative deviation from the normal state (health) of a living organism, where ‘negative’ means an impairment that is measurable in terms of a reduction in the ecological potential (for example survival, growth, reproduction, stress endurance) (Kinne 1980). Thus, disease can be defined as “any process that limits the productivity of a system” (Owens 2003). The great majority of diseases depend on particular interactions between the host, the disease agent or pathogen, and environmental stressors (Austin and Austin 2007); these interactions can be represented in the ‘epidemiological triad’ of Snieszko (Figure 1.4).

Diseases may be divided into infectious and non-infectious. Infectious disease is caused by micro-organisms, including bacteria and fungi. Usually all the components in the ‘epidemiological triad’, i.e. host characteristics (such as fish age, nutritional status,

stocking density), pathogen characteristics (such as its ability to infect, cause disease in and kill the host) and environmental factors (such as poor hygiene, water temperature or salinity) must have to come into play to trigger an outbreak of infectious disease (Owens 2003; Austin and Austin 2007). However, obligate pathogens are independent of other causal factors related to the host and the environment to cause clinical disease, and are regarded as the most hostile agents for disease outbreaks. "Epizootic haematopoietic necrosis virus in red fin perch and crayfish plague (*Aphanomyces astaci*) in signal crayfish are two such examples of obligate pathogens producing disease in the most pristine conditions" (Owens 2003). Moreover, some infectious diseases, e.g. bacterial coldwater disease and infectious haematopoietic necrosis (IHN) in salmon and rainbow trout, cause permanent skeletal deformities or stunting to surviving fish that may account for an indirect production loss (Georgiadis *et al.* 2001). Conversely, non-infectious disease caused by the environment, genetic abnormalities, stress and husbandry practices do not have the ability to spread between separate stocks. Nevertheless, an individual fish may differ in susceptibility to diseases, and also different strains of the same pathogens may vary significantly in their ability to cause a disease (Austin and Austin 2007).

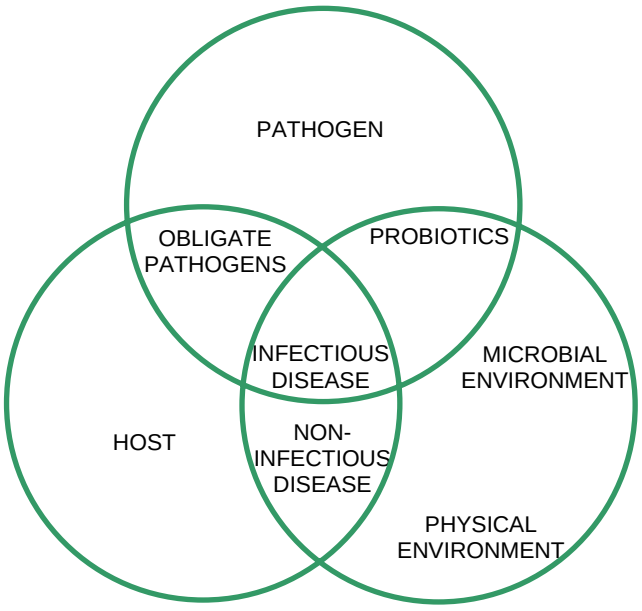

Figure 1.4 The epidemiological triad of Snieszko, indicating the factors of a fish-culture system that can lead to disease (after Owens 2003).

Over the past three decades, aquaculture has expanded, intensified and diversified, and thus numerous aquatic animal diseases and pathogens have emerged and become

widespread especially as a result of global movements of live animals and animal products such as brood stock, seed, and feed (Subasinghe 2005). Certainly, an intensive aquaculture creates the perfect environment to encourage disease because the higher the density the smaller the distance between infected and uninfected fish leading to an increased probability of pathogens reaching new hosts (Owens 2003). Estimates from various organizations have indicated that 30–50% of all fish and shrimp put into cages and ponds are lost due to diseases before they reach market (Tan *et al.* 2006). Thus, the economic losses in the aquaculture industry worldwide are estimated to be about US$8 billion per year, which is roughly 15% of the world's farmed fish and shellfish value (Xie *et al.* 2005). In particular, the outbreaks of viral and bacterial diseases caused losses of about US$750 million in 1993 in China, and US$210 million during 1995–1996 in India (see Kautsky *et al.* 2000) to the penaeid shrimp aquaculture industry, and over US$120 million to the aquaculture industry in China between 1990 and 1992 (Wei 2002).

1.2.1 Bacterial fish diseases

Among the casual agents of infectious diseases, bacteria remain a serious challenge for the growth of global fish and shellfish farming, because major pathogens, such as those causing motile aeromonad septicaemia, vibriosis, columnaris, enteric redmouth disease, edwardsiellosis and furunculosis are prevalent in nature (see Austin and Austin 2007). In northern China, fin rot due to *V. anguillarum* infection in juvenile turbot led to mortalities of 90–100% (Lei *et al.* 2006). Recently, high mortalities have been reported in populations of Atlantic salmon, Pacific salmon and rainbow trout farmed in the South of Chile due to vibriosis (Colquhoun *et al.* 2004; Silva-Rubio *et al.* 2008a,b). Table 1.2 shows the wide variety of bacterial pathogens that have been associated with salmonid fish diseases.

Table 1.2 Bacterial pathogens identified as the most problematic to the salmonids aquaculture (after Austin and Austin 2007).

Pathogens	Disease	Host range	Geographical distribution
ANAEROBES			
Clostridiaceae representative			
Clostridium botulinum	botulism	salmonids	Denmark, England, U.S.A.

GRAM-POSITIVE BACTERIA – THE 'LACTIC ACID' BACTERIA			
Carnobacteriaceae representative			
Carnobacterium piscicola	lactobacillosis, pseudokidney disease	salmonids	North America, U.K.
Enterococcaceae representatives			
Enterococcus (*Streptococcus*) *faecalis* subsp. *liquefaciens*	–	rainbow trout	Italy
Vogococcus salmoninarum	lactobacillosis, pseudokidney disease, peritonitis, septicaemia	Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), rainbow trout	Australia, France, North America
Lactobacillaceae representative			
Lactobacillus spp.	lactobacillosis, pseudokidney disease	salmonids	North America, U.K.
Streptococcaceae representatives			
Lactococcus garvieae (= *Enterococcus seriolicida*)	streptococcicosis/ streptococcosis	many fish species	Japan, Europe, Israel, Saudi Arabia, Red sea, Australia, Taiwan, South Africa, U.S.A.
Lactococcus piscium	lactobacillosis, pseudokidney disease	rainbow trout	North America
Streptococcus difficilis (= *Str. agalactiae*)	meningo-encephalitis	rainbow trout	Israel, Kuwait, U.S.A.
Streptococcus iniae (= *Str. shiloi*)	meningo-encephalitis, streptococcicosis/ streptococcosis	various freshwater and coastal fish species	Australia, Saudi Arabia, Israel, Europe, Bahrain, Japan, South Africa, U.S.A.
AEROBIC GRAM-POSITIVE RODS AND COCCI			
Renibacterium salmoninarum	bacterial kidney disease (BKD; Dee disease; corynebacterial kidney disease)	salmonids	Japan, North and South America, Europe
Bacillaceae representative			
Bacillus spp.	septicaemia, bacillary necrosis	various freshwater fish species	Nigeria, Vietnam
Coryneform bacteria	'corynebacteriosis'	salmonids	England
Micrococcaceae representative			
Micrococcus luteus	micrococcosis	rainbow trout	England
Mycobacteriaceae representatives			
Mycobacterium spp.	mycobacteriosis (fish tuberculosis)	most fish species	worldwide
Nocardiaceae representatives			
Nocardia spp. (*Noc. asteroides*, *Noc. seriolae*, *Noc. salmonicida*)	nocardiosis	most fish species	worldwide

Rhodococcus sp.	ocular oedema	chinook salmon (*O. tshawytscha*)	Canada
Rhodococcus erythropolis	?	Atlantic salmon	Norway, Scotland
Planococcaceae representative			
Planococcus sp.	–	salmonids	England
Staphylococcaceae representative			
Staphylococcus warneri	ulcerations	rainbow trout	Spain
GRAM-NEGATIVE BACTERIA			
Aeromonadaceae representatives			
Aeromonas caviae	septicaemia	Atlantic salmon	Turkey
Aeromonas hydrophila (= *Aer. liquefaciens*, *Aer. punctata*	haemorrhagic septicaemia, motile aeromonas septicaemia, redsore disease, fin rot	many fresh water fish species	worldwide
Aeromonas salmonicida (subspecies *achromogenes*, *masoucida*, and *smithia*) {= *Haemophilus piscium*}	furunculosis, carp erythrodermatitis, ulcer disease	salmonids, cyprinids, dab (*Limanda limanda*), cod	worldwide
Campylobacteriaceae representative			
Arcobacter cryaerophilus	–	rainbow trout	Turkey
Enterobacteriaceae representatives			
Citrobacter freundii	–	salmonids, sunfish (*Mola mola*), carp (*Cyprinus carpio*)	Europe, India, U.S.A.
Edwardsiella tarda (*Paracolobactrum anguillimortiferum*, *Edw. anguillimortifera*)	redpest, edwardsiellosis, emphysematous putrefactive disease of catfish	various freshwater fish species	Japan, Spain, U.S.A.
Escherichia vulneris	septicaemia	various freshwater fish species	Turkey
Hafnia alvei	haemorrhagic septicaemia	cherry salmon (*O. masou*), rainbow trout	Bulgaria, Japan, England
Klebsiella pneumoniae	fin and tail disease	rainbow trout	Scotland
Plesiomonas shigelloides	–	rainbow trout, eel, sturgeon (*Acipenser sturio*), gourami (*Osphyronemus gourami*)	Germany, Portugal, Spain
Serratia liquefaciens	septicaemia	Atlantic salmon, Atlantic charr (*Salvelinus alpinus*), turbot	France, Scotland, U.S.A.
Serratia plymuthica	–	rainbow trout	Poland, Spain, Scotland

Yersinia intermedia	–	Atlantic salmon	Australia
Yersinia ruckeri	enteric redmouth (ERM), salmonid blood spot	salmonids	Australia, Europe, North and South America
Flavobacteriaceae representatives			
Flavobacterium branchiophilum	gill disease	salmonids	Europe, Korea Japan, U.S.A.
Flavobacterium columnare (=*Flexibacter/Cytophaga columnaris*)	columnaris, saddleback disease	many freshwater fish species	worldwide
Flavobacterium hydatis (= *Cytophaga aquatilis*)	gill disease	salmonids	Europe, U.S.A.
Flavobacterium psychrophilum (= *Cytophaga psychrophila*)	coldwater disease, rainbow trout fry syndrome, necrotic myositis	salmonids, sea lamprey (*Petromyzon marinus*)	Australia, Europe, Japan, North America
"*Cytophaga rosea*"	gill disease	salmonids	Europe, U.S.A.
Sporocytophaga sp.	saltwater columnaris	salmonids	Scotland, U.S.A.
Moraxellaceae representative			
Acinetobacter sp.	acinetobacter disease	Atlantic salmon channel catfish (*Ictalurus punctatus*)	Norway, U.S.A.
Moritellaceae representatives			
Moritella marina (= *V. marinus*)	skin lesions	Atlantic salmon	Iceland
Moritella viscosa	winter ulcer disease/syndrome	Atlantic salmon	Iceland, Norway, Scotland
Oxalobacteraceae representative			
Janthinobacterium lividum	anaemia	rainbow trout	Scotland
Pasteurellaceae representative			
Pasteurella skyensis	?	Atlantic salmon	Scotland
Piscirickettsiaceae representative			
Piscirickettsia salmonis	coho salmon syndrome, salmonid rickettsial septicaemia	salmon, sea bass (*Atractoscion noblis*)	Canada, Chile, U.S.A., Greece, Norway, Scotland
Pseudomonaceae representatives			
Pseudomonas anguilliseptica	red spot (= Sekiten-byo), winter disease	rainbow trout, marine fish species, eels (*Anguilla anguilla*, *A. japonica*), black spot sea bream (*Pagellus bogaraveo*), gilthead sea bream (*S. aurata*), cod	Finland, Japan France, Spain, Scotland, Portugal

Pseudomonas chlororaphis	–	amago trout (*O. rhodurus*)	Japan
Pseudomonas flourescens	generalised septicaemia	most fish species	worldwide
Pseudomonas pseudoalcaligenes	skin ulceration	rainbow trout	Scotland
Pseudomonas putida	Haemorrhagic ascites, ulceration	rainbow trout, ayu	Japan, Turkey
Vibrionaceae representatives			
Vibrio anguillarum	vibriosis	most marine fish species	worldwide
V. ordalii	vibriosis	most marine fish species	worldwide
V. logei	skin lesions	Atlantic salmon	Iceland
V. salmonicida	coldwater vibriosis, hitra disease	Atlantic salmon	Canada, Norway, Scotland
V. wodanis	winter ulcer disease/syndrome	Atlantic salmon	Iceland, Norway, Scotland
Miscellaneous pathogens			
'*Candidatus* Arthromitus'	summer enteritic syndrome	rainbow trout	France, Spain
Streptobacillus	–	Atlantic salmon	Ireland
	Varracalbmi	Atlantic salmon	Norway
	ulceration	rainbow trout	Scotland

(–) not associated with a named disease; (?) not associated with specific disease signs

1.3 *Vibrio* infections

Vibriosis due to Gram-negative pathogens of the family Vibrionaceae is one of the most serious bacterial diseases, causing septicaemia and death in a wide range of cold- and warm-water fish species of marine and freshwater origin, and often results in considerable economic losses in fish farming, worldwide (Rodkhum *et al.* 2005; Toranzo *et al.* 2005; Austin and Austin 2007). Generally vibrios are part of the normal marine microflora, and are found in gastrointestinal tract of marine animals or other organisms, and are also associated with live feed organisms, such as rotifers and *Artemia* (Verdonck *et al.* 1997; Eddy and Jones 2002; Mizuki *et al.* 2006; Austin and Austin 2007; Engelsen *et al.* 2008). The genus *Vibrio* has over 85 species (http://www.bacterio.cict.fr), and thus far fifteen types are known to be fish pathogens, namely *V. anguillarum* (= *Listonella anguillarum*), *V. ordalii*, *V. cholerae* (non-01), *V. fischeri*, *V. furnissii*, *V. harveyi* (= *V. carchariae* and *V. trachuri*), *V. ichthyoenteri*, *V.*

logei, *V. pelagius*, *V. salmonicida*, *V. splendidus*, *V. tapetis*, *V. vulnificus*, *V. wodanis* (Austin and Austin 2007). In the present study, *V. anguillarum* and *V. ordalii* were selected as model pathogens because of their well established virulence to salmonids, cod, turbot and halibut (Egidius 1987; Bergh *et al.* 2001; Lillehaug *et al.* 2003; Colquhoun *et al.* 2004; Austin and Austin 2007; Silva-Rubio *et al.* 2008a,b).

1.3.1 Vibrio anguillarum – the classical cause of vibriosis

V. anguillarum possesses a wide geographical distribution and causes a fatal haemorrhagic septicaemia affecting more than 80 different marine and estuarine fish, including species of aquacultural interest, i.e. Pacific and Atlantic salmon (*Oncorhynchus* spp. and *S. salar*), rainbow trout, turbot, sea bass, sea bream, striped bass, cod, Japanese eel (*Anguilla japonica*) and European eel, ayu, saithe (*Pollachius virens*) and Japanese flounder (*Paralichthys olivaceus*) (Actis *et al.* 1999; Kent and Poppe 2003; Colquhoun *et al.* 2004; Chrstiane *et al.* 2006; Austin and Austin 2007; Xiao *et al.* 2009). The pathogen was first described by Bergman in 1909 as the aetiological agent of 'red-pest of eel' during 1907 in Sweden. However, there was a description of red-pest probably dating back to the early 1700s in diseased eel from Italy. Despite the commonly used name of 'vibriosis', the disease has been referred to as 'salt-water furunculosis', 'boil-disease', and 'ulcer-disease' (see Austin and Austin 2007). It should be noted that *V. anguillarum* was suggested to be reclassified as *Listonella anguillarum* in the mid-1980s based on analysis of the 5S rRNA sequence (Macdonell and Colwell 1985), and thus *Listonella* has been on the 'List of Prokaryotic Standing in Nomenclature' since 1986 (http://www.bacterio.cict.fr/l/listonella.html). However, there is still debate regarding this change in nomenclature; consequently both names are used in recent published work (e.g. Gonzalez *et al.* 2003; Mizuki *et al.* 2006; Fjellheim *et al.* 2007; Caipang *et al.* 2008; Sugita *et al.* 2008).

1.3.1.1 Characteristics

V. anguillarum is a halophilic (grows in 0.3–3.0% NaCl) Gram-negative rod-shaped bacterium (0.5–0.8 μm in diameter and 1.4–2.6 μm in length) that is facultatively anaerobic, fermentative, catalase and oxidase positive, and motile by means of a sheathed polar flagellum, with growth between 15–37°C. A positive result is usually recorded for the Voges-Proskauer reaction, but not for the methyl red test. Also, nitrates are reduced. The G+C molar percentage (mol %) of the DNA is 45.6–46.3 (Austin and

Austin 2007). The pathogen can survive in seawater for over 50 months (Crosa *et al.* 2006). Genetically, *V. anguillarum* is a heterogeneous species and so far 23 serotypes are known to occur based on antigen 'O' (Pedersen *et al.* 1999). However, only serotypes O1 and O2, and to some extent serotype O3 are regarded as serious pathogens because most vibriosis outbreaks are related to one of these serotypes (Toranzo and Barja 1990; Knappskog *et al.* 1993; Larsen *et al.* 1994; Larsen *et al.* 2001; Pedersen *et al.* 1999; Silva-Rubio *et al.* 2008b). The remaining serotypes (O4–O23) are considered as environmental strains. However, few of them were described as pathogenic to fish (serotype O4; Pazos *et al.* 1993) or in combination in the mortality of cultured cod (O4, O6 and O8; Larsen *et al.* 1994).

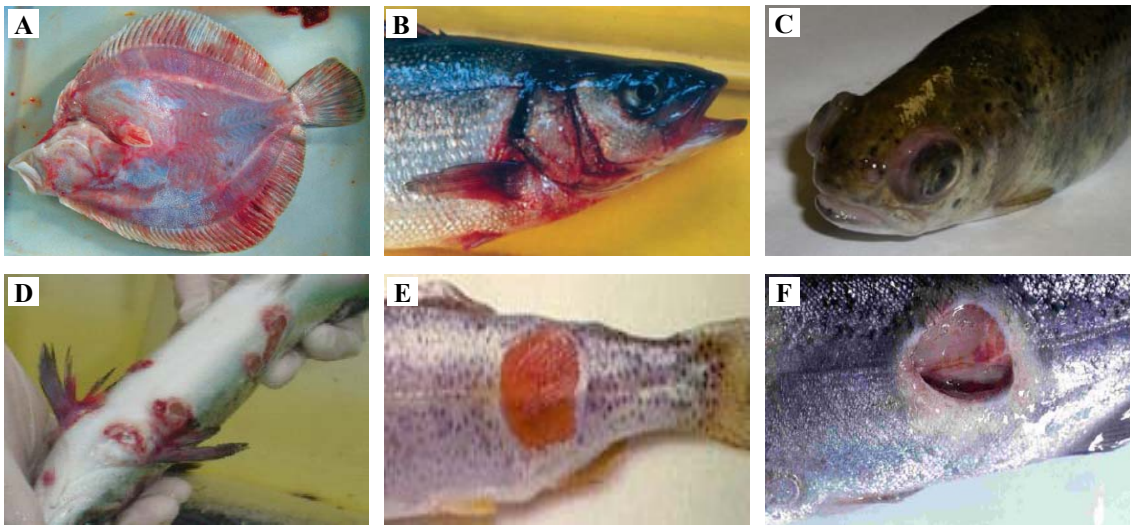

Figure 1.5 Clinical signs of vibriosis: (A, B) surface haemorrhage (source: Austin and Austin 2007), (C) exophthalmia, (D) haemorrhaging ulcers (http://aqua.intervet.com), (E) skin lesions/ulcer (source: http://www.peteducation.com) and (F) muscle necrosis (source: Prof. Lone Gram).

1.3.1.2 Pathology

Infection with *V. anguillarum* is normally characterized by generalized septicaemia with clinical signs identical to other bacterial septicaemias (Figure 1.5). The disease ranges from peracute, i.e. mortalities without gross lesions, to acute, which typically involves skin discoloration, the presence of red necrotic lesions in the abdominal muscle, erythema at different body organs (i.e. bloody blotches at the base of the fins, around the vent and within the mouth), with possibly exophthalmia and fin rot. Internal symptoms include pale liver, accumulation of reddish ascitic fluid in the peritoneal

cavity (= sign of severe infection), swollen spleen, and distended gut and rectum filled with clear viscous fluid. Also, the bacteria's strong affinity for iron may result in anaemia in chronically infected fish (Diggles *et al.* 2000; Austin and Austin 2007). For example, sea bream (*Acanthopagrus cuvieri*) infected by *V. anguillarum* demonstrates necrosis and atrophy of hepatocytes, necrosis of sheathed arteries in the spleen, and necrosis of renal tubules and glomeruli in the kidney (Rasheed 1989). Similarly, infected Pacific salmon fingerlings show pathological signs in the blood, connective tissue, gills, kidney, posterior gastro-intestinal tract, anaemia in the liver, and swelling in the spleen. Generally areas of affected tissues are found with an even distribution of the pathogen, although the greatest cell concentration is in the blood. Infected fish become inactive, cease feeding and suffer mass mortalities (see Austin and Austin 2007). In addition, the extracellular products (ECPs) secreted by the pathogen are toxic and cause comparable pathological changes to those elicited after inoculation of live *V. anguillarum* cells, albeit with the development of lesser acute lesions. It may be that the live pathogen can multiply spontaneously in the kidney and spleen causing more acute lesions to the host (Lamas *et al.* 1994). Typically, clinical signs attributable to a pathogen are dependent on the species and age of fish, and phase of the disease, i.e. acute, chronic, or subclinical carrier (Toranzo *et al.* 2005).

1.3.1.3 Transmission routes

It is widely recognized that pathogens can gain entry to fish through several portals of entry, including skin, gill and the gastro-intestinal tract (= horizontal transmission), and the parental route through eggs (= vertical transmission) (Tortora *et al.* 1995). However, the exact mode of infection of *V. anguillarum* has been debatable, but undoubtedly involves colonization of and attachment to the posterior gastro-intestinal tract and rectum, and then the direct penetration of the tissues (Austin and Austin 2007). For example, the recovery of >50% *V. anguillarum* from the spleen after anal and intragastric intubations (Olsson *et al.* 1996), and colonization of fluorescent-labelled cells in the gastro-intestinal tract with the subsequent development of septicaemia in zebrafish via immersion challenge (O'Toole *et al.* 2004) clearly support the argument of the gastro-intestinal tract as a point of entry. Also, orally administered *V. anguillarum* survived in the stomach of juvenile turbot for several hours, and persisted in the intestine and proliferated in faeces (Olsson *et al.* 1998). Similarly, *V. anguillarum* has been reported to occur in the intestinal epithelium of orally challenged turbot larvae,

where the pathogen was transported to different organs by the blood, leading to death (Grisez *et al.* 1996). These observations evidently demonstrated endocytosis of bacterial cells in the gastrointestinal tract of fish, and indicate that the whole gastrointestinal tract of fish is possibly exposed to infection (Ringø *et al.* 2001). Certainly, adhesion of *V. anguillarum* to rainbow trout by immersion appears to be fastest in the intestinal regions, with maximum attachment occurring within 100 min (Horne and Baxendale 1983). However, using bath immersion as the route of infection, the skin was seen to be colonized at 12 h post infection, followed by the invasion of the liver, spleen, muscle, gills and intestine (see Austin and Austin 2007). Although the gills are mentioned as possible points of entry of *V. anguillarum* (Laurencin and Germon 1987), a few studies revealed poor or unsuccessful transmission (Kanno *et al.* 1989; Olsson *et al.* 1996). In addition, the presence of heavy metals mainly copper (30–60 μg mL^{-1}) and iron (10 μm mL^{-1}) could trigger vibriosis, although susceptibility is dependent on metal concentration and time of exposure (see Austin and Austin 2007).

1.3.1.4 Virulence factors

V. anguillarum is capable of producing many virulence factors that allow colonization and maintenance in the host. High affinity iron uptake mechanisms are recognized as important virulence factors. Indeed, iron is one of the growth limiting factors for all microorganisms, except lactobacilli (see Raaska and Mattila-Sandholm 1995). Additionally, pathogenic bacteria require iron to establish an infection, and thus they have developed siderophores, which is an efficient mechanism for iron acquisition from the host (Ratledge and Dover 2000). Serogroup O1 contains a 67 kbp virulence plasmid pJM1 (Pedersen and Larsen 1995; Di Lorenzo *et al.* 2003), which codes for iron transportation proteins and siderophores (Actis *et al.* 1986) enabling the bacterial cells to acquire available iron in the fish tissues. Thus, invading bacteria may multiply in the host by scavenging for the iron from free heme or heme-containing proteins, such as transferrin, lactoferrin and ferritin, which are present in the serum, secretions and tissues of the fish, respectively (see Austin and Austin 2007). Moreover, acquisition of iron from heme may be assisted by the production of haemolysins or cytotoxins, which have the ability to lyse host cells and release intracellular heme (García *et al.* 1997). Nevertheless, several haemolysin genes are reported in *V. anguillarum* strains (Rodkhum *et al.* 2005; Rock and Nelson 2006). However, serogroups without the pJM1

plasmid are also pathogenic, and are believed to have chromosome-mediated virulence characteristics (Wiik *et al.* 1989).

Other essential virulence mechanisms are attributed to flagella (McGee *et al*. 1996; Milton *et al.* 1996; Ormonde *et al*. 2000), chemotaxis (O'Toole *et al*. 1996; Larsen *et al.* 2004), and ECPs or secreted toxins, e.g. haemolysins, proteases, metalloprotease, dermatotoxin, haemagglutinin and cytotoxin (see Rodkhum *et al.* 2005, Austin and Austin 2007). O'Toole *et al.* (1996) observed that the loss of flagella by transposon mutagenesis led to a 500-fold reduction in virulence following an immersion challenge, which in turn suggests that flagella play a key role in pathogenicity. Besides, chemotactic motility is essential for virulence (Larsen *et al.* 2004), principally by enabling invasion of the host. According to O'Toole *et al.* (1999), the pathogen is attracted to amino acids and carbohydrates predominantly in intestinal mucus and to a lesser degree to skin mucus (see Austin and Austin 2007). Furthermore, chemotaxis was noted when the bacterial cells were starved for 2 and 8 days (Larsen *et al.* 2004). Cells of *V. anguillarum* were more chemotactic to serine (= a mucus component of fish) at 25°C than at 5 or 15°C, and in the salinity range 0.8–2.7% NaCl (Larsen *et al.* 2004). Certainly, *V. anguillarum* is chemotactically motile towards fish skin and intestinal mucus (O'Toole *et al.* 1999), and uses intestinal mucus as a nutrient source (García *et al.* 1997). Moreover, crude ECPs led to the development of an inflammatory response, including leucopenia, in rainbow trout (Lamas *et al.* 1994).

More recently, proteases of 36 kDa molecular weight have been implicated with virulence (Inamura *et al.* 1984; Kanemori *et al.* 1987). In particular, a zinc metallo-protease was found to be associated with invasion (Norqvist *et al.* 1990). In addition, *V. anguillarum* is known to produce 'haemolytic toxins' (see Austin and Austin 2007), which are thermolabile enzymes of 191 kDa and might be responsible for the tissue damage and necrosis, and anaemic response in infected fish. Pathogenicity of *V. anguillarum* may well by attributable to Acylated Homoserine Lactone (AHL) signal molecules, i.e. the quorum sensing. For example, *N*-(2-oxodecanoyl)-L-homoserine lactone and *N*-(3-hydroxy-hexanoyl)-L-homoserine lactone with smaller amounts of other molecules have been recognized in *V. anguillarum*, perhaps having a role in the expression of virulence factors, e.g. biofilm formation and protease production (Buchholtz *et al.* 2006).

Resistance to the potentially debilitating effect of fish serum of this pathogen (Trust *et al.* 1981) can play an important role in pathogenesis, which may accelerate the invasion processes (see Austin and Austin 2007). Also environmental parameters such as the rise in (or rapidly changing) temperatures (above 10°C, i.e. during early to mid summer) and prolonged exposure to low estuarine salinities have been reported as a possible trigger of disease outbreaks (Rodgers and Burke 1981; Austin and Austin 2007). Nevertheless, the virulence mechanism of *V. anguillarum* involves uptake and penetration of the host tissues, scavenging for iron as a result of plasmid/chromosomal-mediated traits, and damage to the fish by means of haemolysins and proteases (Austin and Austin 2007).

Table 1.3 Methods of isolation for *V. anguillarum* and *V. ordalii* (after Crosa *et al.* 2006; Austin and Austin 2007).

Medium			Pathogen
Nutrient agar TSA (tryptone soya agar) BHIA (brain heart infusion agar)	+	supplemented with 0.5–3.5% NaCl (w/v)	*V. anguillarum*
VAM (*V. anguillarum* medium; Alsina *et al.* 1994)			*V. anguillarum*
Seawater agar (marine 2216E agar, Difco)			*V. anguillarum*, *V. ordalii*
TCBS (thiosulphate citrate bile salt sucrose agar)			*V. anguillarum*, *V. ordalii*

1.3.1.5 Isolation/detection

No single technique is suitable for the recovery of all known bacterial fish pathogens, thus researchers need to use a combination of methods and incubation conditions to achieve pure cultures. However, *V. anguillarum* isolation is readily done from infected fish tissue using a range of bacteriological media (Table 1.3), with incubation at 15–25°C for up to 7 days. On solid medium, such as marine 221E agar, the colonies appear glistening cream-colored, circular, raised, entire and shiny (Austin and Austin 2007). The presumptive identification is effectively done by means of *V. anguillarum* medium (VAM) containing bile salts with a high sodium chloride concentration, ampicillin, sorbitol and a high pH (Alsina *et al.* 1994). *V. anguillarum* produces bright-yellow colonies with yellow haloes on VAM, which is identical for the majority, i.e. 197/227 = 87%, of *V. anguillarum* isolates (Austin and Austin 2007). However, growth on VAM is not definitive evidence because some other vibrios (3/66 = 4%) are also mistaken for *V. anguillarum* (Alsina *et al.* 1994; Austin and Austin 2007). Nowadays, the API 20E

system (BioMérieux, France) is used extensively for identification. However, identification of *V. anguillarum* based purely on the results of the API 20E system is not recommended (Grisez *et al.* 1991). There are commercial kits for the specific identification of *V. anguillarum*, such as the Bionor® latex agglutination kit (Romalde *et al*. 1995), AquaRapid-Va® and Aqua-Eia-Va® kits (Gonzalez *et al*. 2004), but they are not recommended for discrimination between serotypes. In addition, molecular techniques including polymerase chain reaction (PCR; Gonzalez *et al.* 2003), pulse field gel electrophoresis (PFGE; Skov *et al.* 1995) amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), repetitive extragenic palindrome PCR (rep-PCR), and ribotyping have been used successfully to identify vibrios, both at the species and strain level (see Thompson *et al.* 2004, 2005).

1.3.2 Vibrio ordalii

V. ordalii is the former biotype 2 of *V. anguillarum*, which was proposed as a separate species in view of cultural and biochemical characteristics as well as DNA homology (Crosa *et al.* 2006). The organism is also recognized as the aetiological agent of vibriosis with gross pathological symptoms comparable to those resulting from infection with *V. anguillarum* (Toranzo and Barja 1993). Disease caused by *V. ordalii* has been documented in Japan and the Pacific Northwest of the U.S.A. (Austin and Austin 2007).

1.3.2.1 Characteristics

V. ordalii comprises Gram-negative, curved rods of 2.5–3.0 × 1.0 μm in size, motile by means of single polar flagellum with fermentative and respiratory metabolism. The bacterium can be separated from *V. anguillarum* by the negative production/results of arginine dihydrolase, β-galactosidase and the Voges-Proskauer reaction, and by growth temperature which is between 15 and 22°C, but not at 37°C. Also the plasmid profile of *V. ordalii* is different from *V. anguillarum* (Austin and Austin 2007). The G+C ratio of the DNA of *V. ordalii* is 43–44 mol %. Characteristically, isolates of *V. ordalii* are homogeneous by plasmid type (pMJ101) with a molecular weight of 20 MDa (= ~32 kbp), ribotyping and serogrouping, accommodated two lipopolysaccharide (LPS) groups, and are heterogeneous by BIOLOG-GN fingerprints and API 20E profiles (Austin *et al.* 1997; Austin and Austin 2007).

1.3.2.2 Pathology

The disease is characterized as a haemorrhagic septicaemia, but there are slight variations in the pathologies compared to that caused by *V. anguillarum*. In the case of *V. ordalii* infection in Pacific salmon, there is a tendency for the formation of micro-colonies in the skeletal and heart muscle, gill tissue, and in both the anterior and posterior regions of the gastro-intestinal tract. Moreover, bacteraemia develops much later in the disease cycle compared to *V. anguillarum*, which may be attributed to the lower bacterial cell numbers in the blood. A different distinguishable feature is the marked reduction of numbers of leucocytes in the blood, i.e. leucopenia (Austin and Austin 2007).

1.3.2.3 Transmission routes

V. ordalii may be prevalent in the environment. So, water-borne infection seems the most likely mode of transmission for *V. ordalii* infestation on fish. Typically, infection (colonization) begins in the rectum and posterior gastro-intestinal tract. Alternatively, its presence on skin suggests that entry may proceed by direct invasion of the integument (Austin and Austin 2007).

1.3.2.4 Virulence factors

The virulence plasmid pJM1, has not been detected in *V. ordalii* (see Austin and Austin 2007). However, a 30 kbp extrachromosomal element (= cryptic plasmid), which was named pMJ101, which replicates in the absence of DNA polymerase I without generating single-stranded intermediates, has been found in all isolates of *V. ordalii* (Bidinost *et al.* 1999; Austin and Austin 2007). Haemolysins and proteases have not been found (Kodma *et al.* 1984). *V. ordalii* induces a pathogenesis not particularly different from that of *V. anguillarum*, but is generally less severe and with sporadic peracute cases. Infection may be via ascending infection from the posterior gut, or through the skin – a phenomenon rarely true outside salmonids (see Hjeltnes and Roberts 1993). The ability of *V. ordalii* to agglutinate trout and human erythrocytes, and yeast cells suggests that it could attach to and interact with the host cells, although this observation was inconsistent in some studies (Crosa *et al.* 2006).

1.3.2.5 Isolation/detection

As with *V. anguillarum*, isolation and identification involves the use of seawater agar and TCBS (Table 1.3) with incubation at 15–25°C for up to 7 days (Austin and Austin 2007). Cultures on marine 2216E agar appear as off-white, circular and convex colonies of 1–2 mm in diameter with slow growth rate (i.e. colony formation takes place between 4–6 days of incubation at 22°C). The biochemical tests frequently used to distinguish between *V. ordalii* and *V. anguillarum* are listed in Table 1.4.

Table 1.4 Phenotypic properties used to differentiate *V. anguillarum* from *V. ordalii* (after Crosa *et al.* 2006).

Biochemical tests and growth temperature	Result for:	
	V. anguillarum	*V. ordalii*
Arginine-alkaline reaction	+	–
Citrate, Christensen	+	–
Citrate, Simmons	+	–
Lipase	+	–
[1]ONPG hydrolysis	+	–
Starch hydrolysis	+	–
Voges-Proskauer reaction	+	–
Acid production from:		
Cellobiose	+	–
Glycerol	+	–
Sorbitol	+	–
Trehalose	+	–
Growth at 37°C	+	–

[1]*o*-Nitrophenyl-β-D-galactopyranoside

1.4 Disease control measures

Sanitary measures (= water quality), better nutrition, lower stocking densities and good farm husbandry practices may successfully prevent the introduction of pathogens in the fish farm environment, including problems attributed to vibriosis (Vendrell *et al.* 2006; Austin and Austin 2007). However, the search for effective disease control/prevention strategies in the last decades has led to some modern approaches, for example, the use of non-specific immunostimulants (e.g. β-1,3 glucan), dietary supplements (e.g. vitamin C), probiotics, the development of genetically disease-resistant stock, and restriction on

the movement of infected stock other than the traditional use of vaccines and antibiotics (Austin 2002; Austin and Austin 2007).

1.4.1 Chemotherapy

Antibiotic/chemotherapeutic agents are probably the most popular methods to treat infectious bacterial diseases, and are applied either as feed additives, by injection, or are added directly to the water (Crosa *et al.* 2006, Austin and Austin 2007). Generally flumequine and oxolinic acid (both quinolones), florfenicol, ampicillin, furanace, furazolidone, nitrofurantoin, oxytetracycline (= terramycin), chloramphenicol (= chloromycetin), nalidixic acid and derivatives, nitrofuran derivatives, kanamycin, sulphonamides and trimethoprim have been used to treat vibriosis with good results (Colquhoun *et al.* 2004, Crosa *et al.* 2006; Austin and Austin 2007). However, differences in sensitivity between serotypes and indeed between cultures of *V. anguillarum* to antibiotics are observed (Table 1.5), and strains have emerged that are resistant towards oxolinic acid (Colquhoun *et al.* 2007) and other antimicrobial compounds (see Crosa *et al.* 2006; Austin and Austin 2007) – a phenomenon well known in *V. anguillarum* (Aoki *et al.* 1985; Pedersen *et al.* 1995). It should be emphasized that antibiotic use in aquaculture may be detrimental to the environment and human health, and involves the development and transfer of resistance to other aquatic bacteria (Petersen *et al.* 2002; Alcaide *et al.* 2005), fish pathogens (Schmidt *et al.* 2000) and human pathogens (WHO 1999; Huys *et al.* 2007), the accumulation of residual antibiotics in aquaculture products (Cabello 2006; WHO 2006), and an effect on the microbial biodiversity (Zhou *et al.* 2009).

Table 1.5 Sensitivity of *V. anguillarum* serotypes O1 and O2 to selected antibiotics used in aquaculture (after Gratacap 2008).

Antibiotic	Serotype O1	Serotype O2
Colistin	Resistant	Sensitive
Ampicillin	Sensitive	Resistant
Cephalothin	Sensitive	Resistant

1.4.2 Vaccines

Prophylaxis measures may not be always effective in controlling diseases. Moreover, once fish are infected they tend to cease daily food intake, which may hinder

chemotherapy applied via feed (Austin and Austin 2007). Thus, vaccination should provoke a protective immune response without causing adverse side effects (Ellis 1989), and ideally should be the best method of disease control. However, only a comparatively few vaccines are available for use in aquaculture (Thorarinsson and Powell 2006). Vaccination of salmonids against vibriosis has proven to be successful (reviewed by Sommerset *et al.* 2005). As a result, the use of antibiotics in the treatment of *Vibrio* infections has been reduced dramatically (Håstein *et al.* 2005; Sommerset *et al.* 2005). Currently, most of the commercial vaccines are derived from simple empirically developed inactivated/killed cultures of bacterial pathogens, namely, *V. anguillarum*, *V. ordalii*, *V. salmonicida* and *Yersinia ruckeri*, being marketed as formalin inactivated vaccines (Gudding *et al.* 1999) either in monovalent (= a single antigen/microorganism) or multivalent/polyvalent (= multiple organisms) forms (see Gudmundsdóttir and Björnsdóttir 2007). Live attenuated vaccines, which are produced from pathogens following the deletion of specific genes known for virulence, cause essentially a controlled infection in the host. A few recombinant subunit vaccines, which are immunogenic protein units, i.e. whole, segments or regions of protein of disease agents or equally active synthetic peptides, are also available and regarded as second generation vaccines. Third generation vaccines are DNA vaccines, which use antigen-encoding plasmid DNA to express an encoded protein and stimulate the host's immune system, are effective against viral fish diseases, such as infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) in rainbow trout, and ictalurid herpes virus 1 (IHV-1) in channel catfish (*Ictalurus punctatus*) (see Verri *et al.* 2003). Currently commercial DNA vaccines are available against infectious pancreatic necrosis virus (IPNV) and IHNV in Norway and Canada (Adams and Thompson 2006).

The majority of commercial vaccines contain adjuvants, substances used to improve the immune response and generally considered to (i) augment the consumption of antigens and the presentation of cells, (ii) induce a 'signal of danger' via pattern recognition receptor signaling, (iii) give a secondary signal (co-stimulatory) in the activation of lymphocytes (Secombes 2008), and (iv) allow an extended delivery of antigens (Evensen *et al.* 2005). Adjuvants used in fish vaccines include oil (which often produce the best results), glucans and aluminium salts (Midtlyng 1996). Vaccines are administered to fish in three different ways: injection (usually intraperitoneally),

immersion and orally; each of these methods has inherent advantages and disadvantages (for details see Gudmundsdóttir and Björnsdóttir 2007). However, vaccination regimes in fish farming do not always offer consistent protection; thus new outbreaks of classical vibriosis in vaccinated fish still occur (Bricknell *et al.* 2006; Samuelsen *et al.* 2006), and occasionally the causative agents appear to be other/new variants of *V. anguillarum* (Mikkelsen *et al.* 2007).

1.4.3 Immunostimulants

An immunostimulant is defined as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" (Bricknell and Dalmo 2005). The approach has been shown to be effective against a range of bacteria and viruses, and is suitable for many species of fish. Moreover, efficacy is often better than that of vaccination (Sakai 1999). A wide range of immunostimulants, over 20 different compounds, has been used in aquaculture (Anderson 1992) and include, substances of microbial origin such as glucan polymers and lipopolysaccharides, extracts from animals and herbal/medicinal plants, nutritional factors (= vitamins), or synthetic compounds namely levamisole and hydroxy-methyl-butyrate, and hormones. Most immunostimulants augment the innate defence mechanisms, but may also enable adaptive immune responses, and may have positive effects on antibody synthesis. Immunostimulants are easy to apply as they can be administered either orally via feeds, by immersion or by injection, and can be readily used with small fish. Potentially, immunostimulants can make cost effective dietary supplements due to the relatively low cost of their source ingredients (Anderson 1992; Sakai 1999; Bricknell and Dalmo 2005; Kunttu *et al.* 2009). Overall, the use of immunostimulants as feed supplements may improve the innate defence of animals providing resistance to pathogenic agents during periods of stress, for example during grading, reproduction, marine transfer and vaccination (Bricknell and Dalmo 2005).

1.4.4 Administration of beneficial bacteria – the probiotics

Nowadays, antibiotic-resistant pathogens and new pathogens are emerging in aquaculture, and the value of prevention of infection is acknowledged (Sanders 2003). Consequently, the use of probiotics, which was initiated during the late 1980s (Kozasa 1986; Dopazo *et al.* 1988; Kamei *et al.* 1988), has garnered attention for disease prevention (Nikoskelainen *et al.* 2001; Aly *et al.* 2008; Pan *et al.* 2008; Vendrell *et al.*

2008). Also, probiotics have been attributed with improved nutrition (Balcázar *et al.* 2006a) and food safety in a more environmentally friendly way (Macey and Coyne 2005). Certainly, FAO has now highlighted the value of probiotics as a means of improving the quality of the aquatic environment (Subasinghe *et al.* 2003).

1.4.4.1 Definition

The concept of probiotic was used by Lilley and Stillwell in 1965 to describe "substances secreted by one microorganism which stimulate the growth of another", thus 'probiotic', which is a Greek word meaning 'for life', is the opposite of the name antibiotic. It was Parker (1974), who probably first introduced the term and definition of 'probiotic' as "organisms and substances which contribute to intestinal microbial balance". Since then the description of probiotics has evolved throughout the years and is commonly used to name bacteria associated with beneficial effects on humans and animals. Fuller (1989) revised probiotics as a "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" – where the idea of substances were omitted as probiotics, but highlighted only the use of live microorganisms. To accommodate the immunostimulation aspect of probiotics, Naidu *et al.* (1999) modified the concept of probiotic as "a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract". In the field of aquaculture, Moriarty (1998) widened the definition of probiotics to microbial 'water additives'. Moreover, Verschuere *et al.* (2000) proposed a broader application of the term probiotic as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". Furthermore, Salminen *et al.* (1999) proposed that a probiotic is "any microbial cell preparation or components of microbial cells that have a beneficial effect on the health and well-being of the host" – where dead cells or components of microorganisms are also included as probiotics if beneficial to the host. So, there is a bewildering array of definitions and the 'probiotic concept' remains controversial, because there were relatively few well controlled studies on the mode of action of probiotics *in vivo* (Atlas 1999). Nevertheless, FAO/WHO (2001) simplified and integrated all theses definitions

and stated that probiotics are "live microorganisms which when administered in adequate amounts, confer a health benefit on the host".

1.4.4.2 Probiotics for use in aquaculture

Many microorganisms have been evaluated as probiotics to improve growth or survival of farmed aquatic species, with candidate probionts often isolated from healthy adult fish (Gildberg *et al.* 1997; Gram *et al.* 1999), rearing water (Lauzon *et al.* 2009) and larvae (Gatesoupe 1999; Ringø and Vadstein 1998). It is interesting to note that human probiotics, i.e. *L. rhamnosus* (Nikoskelainen *et al.* 2003) and *L. plantarum* (Picchietti *et al.* 2007) and beneficial bacteria from terrestrial animals, i.e. *Clostridium butyricum* from the intestine of healthy chicken (Pan *et al.* 2008) offer promise for use in aquaculture. However, the search for new microorganisms continues. To date, a number of probiotic bacteria have been used in aquaculture, and these are mainly derived from two bacterial divisions, (i) Gammaproteobacteria, such as *Aeromonas*, *Pseudomonas*, *Shewanella* and *Vibrio*, and (ii) Firmicutes including lactic acid bacteria (= *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Weissella*), *Bacillus* and *Clostridium*. The other phylogenetic lineages are Actinobacteria (= *Arthrobacter*, *Micrococcus*) and Alphaproteobacteria (= *Roseobacter*) – Table 1.6.

Table 1.6 Probiotics used in aquaculture of fishes.

Probiotics	Source	Host species	References
GRAM-POSITIVE BACTERIA			
Arthrobacter sp.	cod larval rearing water	Atlantic cod	Lauzon *et al.* (2009)
Bacillus sp.	common snook	common snook (*Centropomus undecimalis*)	see Irianto and Austin (2002b)
B. subtilis	digestive tract of rainbow trout	rainbow trout	Newaj-Fyzul *et al.* (2007)
B. megaterium, *B. subtilis*, *B. polymyxa*, *B. licheniformis*	commercial product	channel catfish	Queiroz and Boyd (1998)
B. circulans	intestines of rohu	rohu (*Labeo rohita*)	see Balcázar *et al.* (2006a)
Carnobacterium sp.	intestine of rainbow trout, Atlantic salmon, rotifers	rainbow trout/fry, Atlantic salmon, turbot larvae	Irianto and Austin (2002a); Robertson *et al.* (2000)

C. divergens	intestine of Atlantic salmon	cod	Gildberg *et al.* (1997); Gildberg and Mikkelsen (1998); Kim and Austin (2006a)
C. inhibens	intestine of Atlantic salmon	salmonids	see Irianto and Austin (2002b)
C. maltaromaticum	rainbow trout	rainbow trout	Kim and Austin (2006a)
Clostridium butyricum	intestine of chicken	Chinese drum (*Miichthys miiuy*)	Pan *et al.* (2008)
Enterococcus sp.	algal supplement	Atlantic cod	Lauzon *et al.* (2009)
E. faecium	commercial product, piglet	eel, tilapia (*Oreochromis niloticus*)	Chang and Liu (2002); Wang *et al.* (2008)
Lactobacillus sp.	rotifers, tilapia intestine	turbot larvae, tilapia	Gatesoupe (1994); see Irianto and Austin (2002b)
L. fructivorans	Sea bream gut	Sea bream	Carnevali *et al.* (2004)
L. plantarum	human faeces, turbot larvae	Sea bream, turbot	Carnevali *et al.* (2004); see Irianto and Austin (2002b)
L. bulgaricus	?	turbot larvae	see Balcázar *et al.* (2006a)
L. rhamnosus	culture collection	rainbow trout	Nikoskelainen *et al.* (2001); Panigrahi *et al.* (2004)
L. helveticus	turbot larvae	turbot	see Irianto and Austin (2002b)
Leuconostoc mesenteroides	salmonids	rainbow trout	Vendrell *et al.* (2008)
Micrococcus luteus	digestive tract of rainbow trout, Nile tilapia	rainbow trout, Nile tilapia	Irianto and Austin (2002a); El-Rhman *et al.* (2009)
Pediococcus acidilactici	commercial product	pollack larvae (*Pollachius pollachius*)	Gatesoupe (2002)
Streptococcus lactis	?	turbot larvae	see Balcázar *et al.* (2006a)
S. thermophilus	turbot larvae	turbot	see Irianto and Austin (2002b)
Weissella helenica	flounder intestine	flounder (*Paralichthys olivaceus)*	see Irianto and Austin (2002b)
GRAM-NEGATIVE BACTERIA			
Aeromonas hydrophila	digestive tract of rainbow trout	rainbow trout	Irianto and Austin (2002a)
Aer. caviae	turbot larvae	turbot larvae	Ringø and Vadstein (1998)
Pseudomonas sp.	rainbow trout, Nile tilapia	rainbow trout, Nile tilapia	Spanggaard *et al.* (2001); El-Rhman *et al.* (2009)
P. fluorescens	iced freshwater fish, brown trout, rainbow trout	rainbow trout, Atlantic salmon	Gram *et al.* (1999); see Irianto and Austin (2002b); Gram *et al.* (2001)
P. chlororaphis	intestine of perch	juvenile perch (*Perca fluviatilis*)	Gobeli *et al.* (2009)

Roseobacter sp.	turbot larvae rearing farm	turbot larvae	Hjelm *et al.* (2004)
Shewanella sp.	seabream skin	Sea bream	Salinas *et al.* (2006)
Vibrio sp.	turbot larvae	turbot	Gatesoupe (1997)
V. fluvialis	digestive tract of rainbow trout	rainbow trout	Irianto and Austin 2002a
V. pelagius	turbot larvae	turbot	Ringø and Vadstein (1998)
V. alginolyticus	beach sand	Atlantic salmon	Austin *et al.* (1995)

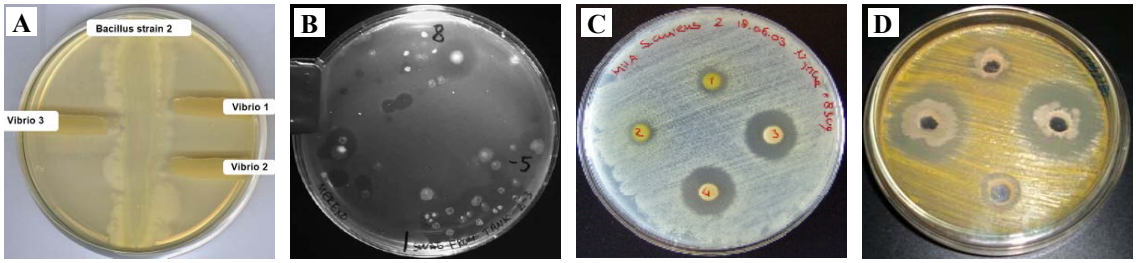

Figure 1.6 Evaluation of the inhibitory activity of putative probiont against undesirable microbes – (A) cross-streak method (Decamp *et al.* 2008), (B) double-layer method (Hjelm *et al.* 2004), (C) disc-diffusion method (http://mason.gmu.edu) and (D) agar well-diffusion method (http://www.ispub.com).

1.4.4.3 Development of novel probiotics

The range of potential probiotics is diverse (Verschuere *et al.* 2000; Hong *et al.* 2005), and includes bacteria (Verschuere *et al.* 2000; Gram and Ringø 2005), bacteriophages (Nakai and Park 2002), yeasts (Tovar *et al.* 2002) and microalgae (Austin *et al.* 1992). A reasonable approach to isolate candidate probionts could be to search among the intestinal microbiota of healthy animals on the assumption that this is the natural location of "good" micro-organisms (Gullian *et al.* 2004). These micro-organisms should be (i) non-pathogenic to the host, (ii) compete with or inhibit the growth of fish pathogens, (iii) adhere to and grow inside the host, and (iv) be indigenous to the environment to which they will be subjected (Gatesoupe 1999; Verschuere *et al.* 2000). The focus of this thesis has been to develop bacteria as probiotic organisms, thus putative probionts were isolated from the host intestine before examining for antibacterial activity against pathogens. Studies have demonstrated that naturally occurring bacteria, i.e. the indigenous microbiota of fish or the rearing environment may produce a range of antagonistic compounds and inhibit the growth of pathogens (Robertson *et al.* 2000, Spanggaard *et al.* 2001, Vine *et al.* 2004a; Fjellheim *et al.* 2007). Such organisms may be invaluable as probiotics (see Hai *et al.* 2009). Several

inhibitory assays have been used, such as the cross-streak, double-layer, spot-on-lawn, disc-diffusion, well-diffusion and co-culture methods – Figure 1.6 (Irianto and Austin 2002a; Brunt and Austin 2005; Hai *et al.* 2007). The cross-streaking method (= bacteriocin-like inhibitory substance) is regarded to be suitable and allows a comparison of inhibitory activity for different putative probionts (Hai *et al.* 2007). This process may involve examination of possible harmful effects of the selected bacterial isolates on the host, preferably by intramuscular/intraperitoneal injection. If non-pathogenic, the organism is evaluated *in vivo* against the selected pathogen choosing a delivery method (see Section 1.4.4.4 for details). Finally, the beneficial strain is identified as probiotic (Irianto and Austin 2002a; Brunt and Austin 2005).

1.4.4.4 Delivery methods

In aquaculture, probiotic cultures have been applied either as food supplements or as additives to water (Moriarty 1998), and include (i) bathing the host in bacterial suspensions, (ii) addition to culture water, or (iii) supplemented with diets, i.e. simply mixed with inert diet or enrichment of live food (artemia/rotifers). Most studies have administered the probiotics to fish or shellfish (larvi)culture by adding them to the diet or directly into the rearing media; the former being more practical than the latter in reality (Hai *et al.* 2009). A feed supplement of *Leuconostoc mesenteroides* and *L. plantarum* dosed at 10^7 colony forming units (cfu) g^{-1} for 30 days (Vendrell *et al.* 2008), and a mixture of *B. subtilis* and *B. licheniformis* dosed at 4×10^4 spores g^{-1} for 42 days (Raida *et al.* 2003) controlled infections caused by *Lactococcus garvieae* and *Y. ruckeri* in rainbow trout. Similarly, the application of probiotics via rearing water has been successful (Queiroz and Boyd 1998; Ringø and Vadstein 1998; Ottesen and Olafsen 2000; Makridis *et al.* 2008). Riquelme *et al.* (1997) reported a reduction in mortality following the bathing of scallop (*Argopecten purpuratus*) larvae in probiotic suspension (Suomalainen *et al.* 2005). Certainly, it is necessary to optimize the methods of delivery to the host, including consideration of dose (Brunt and Austin 2005; Son *et al.* 2009), feeding duration (Aly *et al.* 2008), and composition of the preparation, namely single- or multiple strains (Aly *et al.* 2008; Hai *et al.* 2009).

1.4.4.5 Possible mode of action

The relationship between probiotic bacteria and their hosts is very complex, and the mechanisms of action of probiotics are not completely understood. However, the effect on reducing disease may be linked to a combination of factors (Figure 1.7):

(i) host immunostimulation, i.e. enhancement of humoral and cellular immune response (Irianto and Austin 2002b; Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2005; Salinas *et al.* 2005; Diaz-Rosales *et al.* 2006),

(ii) inhibition of or competition with potential pathogens (= production of antibiotics, and competition for adhesion sites or nutrient/energy sources) (Gram *et al.* 1999; Verschuere *et al.* 2000; Irianto and Austin 2002b; Vine *et al.* 2004b; Chabrillón *et al.* 2005b; Hong *et al.* 2005; Balcázar *et al.* 2006a; Brunt *et al.* 2007; Decamp *et al.* 2008),

(iii) enhancement in feed efficiency, source of nutrients or enzymatic contribution to digestion (Tovar *et al.* 2002; Balcázar *et al.* 2006a; Tinh *et al.* 2008; Sáenz de Rodrigáñez *et al.* 2009),

(iv) improvement of water quality (Verschuere *et al.* 2000; Taoka *et al.* 2006a; Lallo *et al.* 2007), and

(v) improvement in the microbial balance (Hill *et al.* 2009; Picchietti *et al.* 2009).

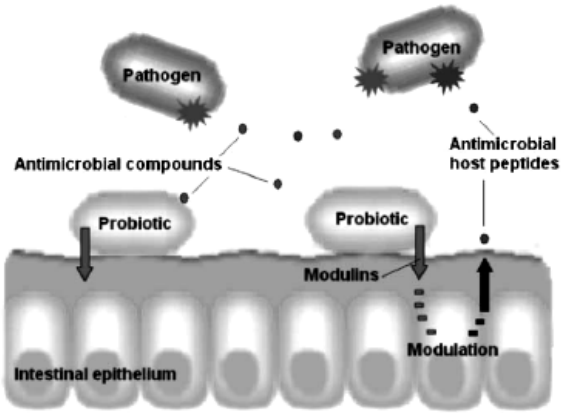

Figure 1.7 Possible mode of action of probiotics in the intestinal tract of a host (source: Balcázar *et al.* 2006b).

1.5 Defense mechanisms of fish

Fish are poikilothermic aquatic vertebrates, which possess a system of defence mechanisms closer to mammals than to any invertebrate, and involve elements of both innate and specific immunity (Ellis 1982; Alvarez-Pellitero 2008). The innate mechanism of fish provides the first line of immune defence. The specific immune mechanisms conferring acquired resistance to disease demand adaptive processes within the immune system forms the second line of defence. There are fundamental differences between the two systems in terms of receptor types used to recognize pathogens. Innate

immune recognition relies on a growing number of pathogen recognizing receptors (PRR), with broad specificity, which are conserved pathogen-associated molecular patterns (PAMPs), such as polysaccharides, LPS, peptidoglycans, β-glucans, bacterial DNA and double stranded viral RNA, and other molecules not normally found on the surface of multicellular organisms (see Magnadóttir 2006; Alvarez-Pellitero 2008). The PRR can be either soluble (such as mannan-binding lectin or C-reactive proteins) or cellular (Mannose receptor or Toll-like receptors, TLRs). For example, C-type lectins are well-known for their capacity to identify specific pathogen-associated carbohydrate structures (Alvarez-Pellitero 2008). On the contrary, adaptive immune recognition is negotiated by antigen (Ag) receptors, with random but narrow specificities (see Alvarez-Pellitero 2008). However, there is increasing evidence of inter-dependency of the different immune mechanisms into a complex multilevel network (also referred to as a combinatorial system), which confronts the dichotomic view between innate and specific immune system (Magnadóttir 2006; Whyte 2007; Alvarez-Pellitero 2008). For example, there are functional as well as genetic proofs that fish have a network of signalling molecules, cytokines and chemokines, that control and co-ordinate the innate and specific immune responses (Secombes *et al.* 1999; Secombes 2002).

1.5.1 Innate immune mechanisms

The innate (= natural or non-specific) immunity includes all of the defence mechanisms that protect an organism against infection, without depending upon prior exposure to any pathogens (Bols *et al.* 2001). It responds to a pathogen in the first few hours after infection (Dixon and Stet 2001). Therefore, this arm of the immunity has been regarded as an essential component in combating disease events. Key innate immune components include the scales, epithelial tissue lines, gill, mucus, the constituents of the blood, phagocytic cells, and proteins mediating the responses, such as complement, which initiates inflammation, or cytokines that control the cellular components (Dixon and Stet 2001; Reynaud *et al.* 2008).

1.5.1.1 Physical and chemical barriers

The epidermis defines the physical separation between the internal and external environment of fish. Thus, the scales, mucus layer on the skin and mucus membranes lining the viscera hollow of the respiratory tract, genito-urinary tract, gills and digestive system act as the first defence against infection (see Bols *et al.* 2001; Magnadóttir 2006;

Reynaud *et al.* 2008). The mucus, which is composed of water and gel-forming macromolecules including mucins and other glycoproteins, is a well known biological barrier to the entry of pathogens from the environment into the fish (Chen *et al.* 2008). By being continuously produced and sloughed off, mucus prevents the adherence of pathogens (Austin and Mcintosh 1988; Subramanian *et al.* 2007), and simultaneously serves as a repository of numerous innate immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive proteins, proteolytic enzymes, flavoenzymes, Apolipoprotein A–I and antimicrobial peptides that are constitutively expressed to provide immediate protection from opportunistic pathogens. In particular, proteases of fish mucus, for example serine-trypsin, cysteine-cathepsin B and L, aspartic-cathepsin D and metallo-proteases, can cleave bacterial proteins, thus possess bacteriolytic activity (see Subramanian *et al.* 2007, 2008).

1.5.1.2 Humoral parameters

Humoral factors (= noncellular nonspecific defence mechanisms), which are found free in the serum or body fluids, are predominantly proteins or glycoproteins, and include lysozyme, peroxidases, anti-proteases (= clearance of active proteases from the tissue fluids), acute-phase proteins, components of the complement pathways, agglutinins and precipitins (opsonins, primarily lectins), transferrin (iron binding protein), cytokines, chemokines, natural antibodies and antibacterial peptides, which non-specifically inhibit the growth of or directly destroy microbial pathogens (Bols *et al.* 2001; Magnadóttir 2006; Whyte 2007; Alvarez-Pellitero 2008; Swain and Nayak 2009). Lysozyme disrupts the cell walls of bacteria by splitting glycosidic linkages in the peptidoglycan layers (Bols *et al.* 2001). Functions of acute-phase proteins, which are plasma or serum proteins (i.e. C-reactive protein, serum amyloid A, ceruloplasmin, fibrinogen), include the regulation of phagocytosis and activation of the classical complement cascade in response to tissue damage, infection, or inflammation (Bayne and Gerwick 2001; Bols *et al.* 2001). Complement, including the alternative, lectin and classical pathways, is among the main mechanisms implicated in the introduction of the innate response and further mounting of an adaptive response (Alvarez-Pellitero 2008). It plays important roles in the lysis of pathogens, by opsonization and activation of phagocytes, and in inflammation (see Alvarez-Pellitero 2008). However, the two subsets of the innate immune mechanisms (the humoral and cellular) do not function in isolation, and co-

operate in many instances in the discharge of their functions. An example is the opsonisation through complement/C-reactive protein/phagocytosis (Ellis 1986).

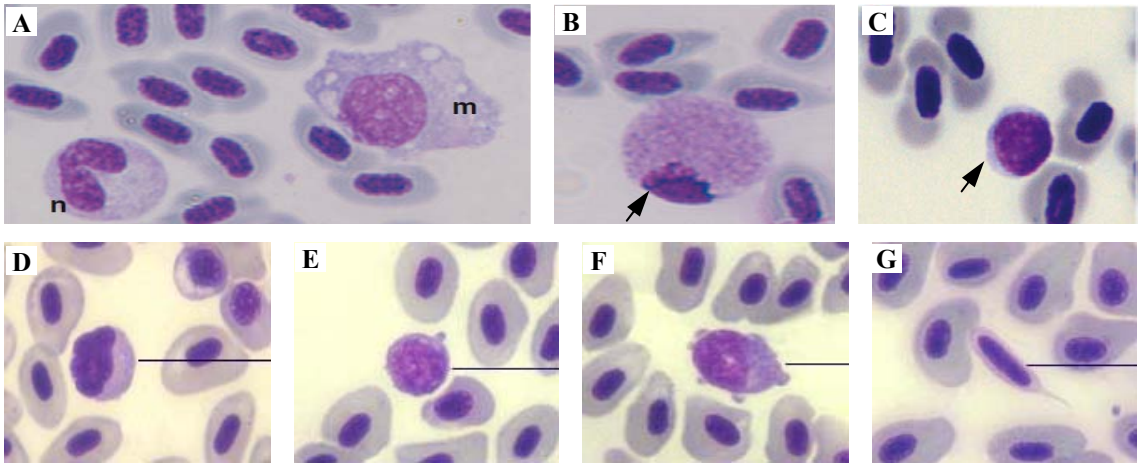

Figure 1.8 Blood cells of fishes: (A) neutrophil granulocyte (n) with segmented nucleus and pale cytoplasm, and monocyte/macrophage (m) with vacuolated cytoplasm, (B) eosinophil with characteristic peripheral non-segmented nucleus and granule-rich cytoplasm, (C) Lymphocyte with characteristic round nucleus and thin blue basophilic cytoplasm (source: Lieschke and Trede 2009), (D) granulocytes, (E) lymphocytes, (F) monocytes, and (G) thrombocytes (source: http://www.aqualex.org).

1.5.1.3 The cells of the innate system

Circulating leukocytes together with the nonspecific cytotoxic cells (NCC) and reticuloendothelial system (RES) form the cellular arm of innate immunity in fish (Secombes 1996; Bols *et al.* 2001). A central feature of the cellular element of innate immunity is its non-specificity, which allows large populations of cells to be mobilized quickly at local and/or systemic sites, after antigenic stimulation (Whyte 2007). Phagocytic cells (= granulocytes, monocytes and macrophages, neutrophils; Figure 1.8) are specialized for the pursuit, capture, ingestion and intracellular destruction of invading microbes, i.e. by phagocytosis. Inducible potent microbiocidal responses, i.e. the respiratory burst (superoxide O_2^-), reactive oxygen species (hydrogen peroxide H_2O_2, hypochlorous acid HOCl, hydroxyl radical OH, singlet oxygen $^1O_2$) and nitric oxide (NO) have been demonstrated in fish phagocytes (see Bols *et al.* 2001; Alvarez-Pellitero 2008). NCC, which mediate the acute innate cytotoxic responses, provide resistance against tumor development, and have been assayed by their capacity to spontaneously lyse certain fish and mammalian tumor cells, some virus-infected fish

cell lines, and specific protozoan parasites (see Bols *et al.* 2001). The RES, which are made up of endothelial cells and macrophages lining the blood vessels, as well as sinusoids and ellipsoids of the spleen and sinusoids of the kidney and liver, take up and degrade microbes (see Bols *et al.* 2001). Nevertheless, cellular immunity develops from an enhanced ability of a particular cell/tissue to counter infection (Cruickshank 1965).

1.5.2 Specific immune mechanisms

In contrast, the specific immune response targets pathogen-specific features, and turns out once the pathogen is present for a defined amount of time in a form and quantity sufficient to stimulate a response by the host (Dixon and Stet 2001). It normally takes many days to develop (Dixon and Stet 2001) because of restrictions put on the specific immune response by their poikilothermic character plus the directories of limited antibodies, affinity maturation and memory and relatively slow proliferation of lymphocytes (Magnadóttir 2006). Temperate species, such as salmonids, require at least 4–6 weeks while cod takes 10–12 weeks to show specific immune responses (see Subramanian *et al.* 2008). For these reasons, this type of immunity is known as acquired or adaptive immunity (Dixon and Stet 2001). Consequently, fish are likely to rely highly on their innate immune mechanisms for protection against invading pathogens. However, lymphocytes, which recognise antigen–MHC (major histocompatibility complex) molecules through surface receptors, are the key effector cells of adaptive immunity contributing to a more specific and efficient response against infections (Reynaud *et al.* 2008; Lieschke and Trede 2009). These cells are able to separate into T- or B-lymphocytes, and usually occur in the peripheral organs (spleen and kidney) and in the circulatory and other tissues monitoring the body for non-self antigens or pathogens (Trede and Zon 1998). The cell-mediated response relies on the presence of accessory cells (= any phagocytes, or professional antigen presenting cells, such as the dendritic cells of mammals) to present antigen to T-cells, which is the mainstay of adaptive cellular immunity (Lieschke and Trede 2009; Swain and Nayak 2009). In addition, there can be various types of humoral activity referring to responses mediated by antibody where B-cells are at the centre (Lieschke and Trede 2009). For example, antibodies can induce responses that are inflammatory, lytic, phagocytic or allergic (see Dixon and Stet 2001). An important molecule in the humoral immunity is immunoglobulin (Ig). An overview of fish immune systems is given in Table 1.7.

Table 1.7 Components and specializations of fish immune systems (as demonstrated by zebrafish); source: Lieschke and Trede (2009).

	Innate immunity	Adaptive immunity
Phylogeny	Many mechanisms shared with all animals; some mechanisms are also shared with plants	Jawless fish have a form of lymphocyte and divergent type of rearranging immune receptor Jawed fish mark the appearance of the thymus and recombinase-activating genes (RAGs)
Anatomical features	Surface barrier with mucus coating containing secreted protective antibacterial molecules Cells and soluble factors distributed in endothelium-lined circulation	No lymph nodes or germinal centers Lymphatic circulation Secondary lymphoid organs: paired thymic organ, anterior and posterior kidney, spleen, gut-associated lymphoid tissue
Key cell types	Macrophages Granulocytes: neutrophil, eosinophil, basophil Natural killer cell equivalent (diverse genomic library of novel immune type receptors, NITRs) Thrombocytes (work with coagulation pathways to maintain host integrity)	B-lymphocytes (re-arranging B-cell receptor) T-lymphocytes (re-arranging T-cell receptor) Detailed lymphocyte subtypes not yet characterized Antigen-presenting cells
Cellular immunity	Phagocytosis (predominantly macrophages) Microbiocidal/static biochemical pathways (e.g. peroxide, nitric oxide production) Hematopoietic growth factors regulate leukocyte precursor proliferation, leukocyte function	Compared to tetrapods, conserved T-cell receptor structure Specificity diversification by RAG-dependent V(D)J recombination Characteristic cytotoxic T-cell responses demonstrable, e.g. mixed lymphocyte reaction, graft rejection, antiviral response
Humoral immunity	Complement – classical, alternative and mannose-lectin pathways; highly evolved diversity Coagulation pathways Natural antibodies Induced cytokines, e.g. interleukin-1, tumor necrosis factors, interferons, chemokines Ligand and receptor families characterised by highly evolved diversity	Compared to tetrapods, less conserved B-cell receptor structure Immunoglobulin classes (μζδ isotypes) Specificity diversification by RAG-dependent V(D)J recombination; somatic hypermutation contributes to a lesser extent No class switching

Activation and key signaling pathways	Pathogen-associated molecular patterns (PAMPs) interact with pattern recognition receptors (PPRs), e.g. Toll-like receptors (TLRs), lectins, peptidoglycan recognition proteins, cytokine receptors	Immune receptor engagement with antigen
Immunological memory	Fixed repertoire, entrenched in the genome	Adapts to environmental history of the individual; mechanisms for adaptation entrenched in genome
Cellular ontogeny	Phagocytes are present and functional from early segmentation	Lymphocytes appear first during late organogenesis
	Haematopoiesis has primitive and definitive phases, at distinct locations during development	T-lymphocytes precede B lymphocytes
	Adult haematopoiesis in the kidney interstitium (not bone marrow)	T-lymphopoiesis resides in the thymus throughout life
		B-lymphopoiesis resides in adult kidney interstitium (embryonic location not certain)
Comparison with mammals	Appears highly evolved and richly diversified	Appears less highly evolved and possibly less flexible

1.6 Aims and objectives

The aim of this study was to develop probiotics to protect rainbow trout from vibriosis.

The specific aims were, as follows:

i) to identify candidate probionts from the intestine of fish useful as control agents against *V. anguillarum* and *V. ordalii* diseases

ii) to assess *in vivo* the effectiveness of the putative probiotics administered to rainbow trout in controlling experimental vibriosis

iii) to establish effective dose(s) rate, feeding duration and composition (= single- or multi strain/species) of probiotics for preventing *Vibrio* infection

iv) to examine the duration of protecton after cessation of feeding with dietary probiotic

v) to study the efficacy of the cell components of probiotics to confer protection

vi) to determine the possible mode of action(s) of probiotics on the host, i.e. on the immunological, growth and enzymatic performances of the host.

CHAPTER 2 – MATERIALS & METHODS

2.1 Fish stocks

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) and Atlantic salmon (*Salmo salar*) of 10–15 g average weight were obtained from a commercial fish farm in Scotland. The fish were maintained in continuously aerated free-flowing dechlorinated freshwater at ~12°C, and fed with commercial pelleted diet (Skretting, Glasgow, Great Britain) at ~2% of body weight daily. The fish had neither been vaccinated nor exposed to fish diseases, and the health of the fish (= changes in physical appearance and internal organs followed by swabs from body surface, kidney and liver for bacteriology) was randomly checked initially upon receipt and then at 2–4 week intervals (after Austin and Austin 1989).

2.2 Fish pathogens

The pathogens, *Vibrio anguillarum* and *V. ordalii*, which were originally recovered from diseased salmonids in Tasmania and Norway, respectively (see Austin *et al.* 1995), were obtained from the fish pathogen collection of the School of Life Sciences, Heriot-Watt University. The cultures were routinely grown on tryptone soya agar (TSA; Oxoid, Basingstoke, Great Britain) plates and in tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) sodium chloride (NaCl; BDH, Poole, Great Britain) referred to as TNA and TNB, respectively, with incubation at 26°C for 18 h. Stock cultures were stored in TNB containing sterile (121°C for 15 min) 20% (v/v) glycerol (Sigma-Aldrich, Basingstoke, Great Britain) at −70°C. For the preparation of challenges, loopfuls of pure cultures from TNA plates were grown in 10 mL of TNB at 26°C for 18 h. Then, cells were harvested by centrifugation (2,500 × *g*, 15 min, 4°C) in a Mark IV refrigerated centrifuge (Baird and Tatlock, London, Great Britain), washed twice in saline and redissolved in fresh saline to achieve the required number of cells mL^{-1} as determined using a haemocytometer slide (Improved Neubauer Type; Merck, Lutterworth, Great Britain) on a Kyowa light microscope (Tokyo, Japan).

2.3 LD_{50} dose of the pathogen

A lethal dose for 50% of the population (LD_{50}) was calculated by the Probit method of Wardlaw (1985) for pathogens *V. anguillarum* and *V. ordalii*, which were used in the challenge model. Thus, groups of 5 rainbow trout were injected intraperitoneally (i.p.) and intramuscularly (i.m.) with 0.1 mL $fish^{-1}$ of pathogen suspensions in saline ranging

from 10^2, 10^3, 10^4, 10^5, and 10^6 cells mL^{-1}, prepared as above, and mortalities recorded over a period of 7 days.

2.4 The development of probiotics

An approach to isolate candidate probionts could be to look among the intestinal microbiota of healthy animals while supposing that this is the natural place of the "good" micro-organism (Gullian *et al.* 2004). Thus, the gut microbiota of healthy rainbow trout and salmon were studied for the development of probiotics (after Irianto and Austin 2002a; Brunt and Austin 2005).

2.4.1 Isolation of putative probionts

Fish were killed by an overdose of anesthetic (MS-222; Sigma-Aldrich). The surface of the fish was sterilised using 70% ethanol (BDH), and the peritoneal cavity was opened aseptically with a sterile scalpel. The intestine between the pyloric caecae and approximately 1 cm anterior to the anus was excised, the intestinal content was removed by gentle squeezing with a sterile forceps and collected into 10 mL of 0.9% (w/v) sterile saline. Ten-fold dilutions were prepared up to 10^{-6} in fresh saline, and 0.1 mL of each dilution was spread onto duplicate plates of TNA. All the plates were incubated aerobically at ambient temperature (~20°C) for a week. Then, colonies were picked randomly, purified by streaking and re-streaking onto fresh TNA plates.

2.4.2 Pathogen inhibition assay

The antimicrobial properties of the isolates, either by overgrowing or interrupting the growth of *V. anguillarum* and *V. ordalii* were tested in order to screen for the potentially beneficial cultures.

2.4.2.1 The cross-streaking method

The antagonistic activity by a random group of 100 colonies was assessed by the cross-streaking method (= bacteriocin-like inhibitory substance) according to Robertson *et al.* (2000). Briefly, a pure colony of the pathogen(s) was picked with a sterile wire loop and inoculated in parallel streaks onto fresh TNA plates. Then, test isolate(s), picked as before, were streaked perpendicularly across the inocula of the pathogen(s). All plates

were incubated at room temperature for up to 7 days whereupon interruption or overgrowth of the pathogen was recorded as evidence of antagonism.

2.4.3 Safety of isolates in fish

Isolates, which demonstrated antagonistic activity against *V. anguillarum* and *V. ordalii* were retained and evaluated further for the lack of harm in rainbow trout. Thus, the isolates were grown in TNB at 26°C for 18 h, cells were harvested by centrifugation (2,500 × *g*, 15 min, 4°C), washed twice in saline and resuspended in fresh saline to ~10^8 cells mL^{-1} as determined by use of a haemocytometer slide and light microscopy. Then, volumes of 0.1 mL $fish^{-1}$ were injected i.p. and i.m. into groups of 10 rainbow trout. Controls were injected with 0.1 mL of saline. The groups of control and treated fish were observed daily for 2 weeks. Challenge experiments were maintained in static, aerated dechlorinated freshwater at ~18°C with ~50% water exchange daily.

2.4.4 Experimental diets

A pure culture of putative probiotic from overnight growth on a TNA plate was inoculated into 10 mL of TNB with incubation at 26°C for 18 h. The culture was centrifuged (2,500 × *g*, 15 min, 4°C), the cell pellet washed twice, resuspended in saline, and the concentration adjusted to ~5 × 10^7 cells mL^{-1}, as above. Then, 50 g quantities of commercial rainbow trout feed were mixed with the bacterial suspension in a glass bottle to achieve a dose of ~5 × 10^7 cells g^{-1}, which was determined as an effective feeding dose by Brunt and Austin (2005) and Kim and Austin (2006a). The modified feed was stored in screw-top glass bottles at room temperature. A control diet contained an appropriate volume of saline instead of the bacterial suspension. The viability of bacteria in the feed was monitored by the total viable count (Nikoskelainen *et al.* 2003). Briefly, 1 g of feed was homogenized in 9 mL of saline using a sterile disposable homogenizer (VWR, Poole, Great Britain), and 10-fold dilutions prepared to 10^{-6} in fresh saline, before 0.1 mL volumes were spread over TNA plates. Colony counts were determined after incubation at room temperature for one week.

2.4.5 Palatability of bacteria-supplemented feed

Putative probiotic bacteria with no harmful effects were orally administered to fish as dietary supplement to determine palatability. For this, separate groups of 10 rainbow

trout were fed to satiation twice a day for 1 week with the experimental (dose = ~5 × 10^7 cells g^{-1}) and control diets. The overall feeding response was observed, i.e. the number of uneaten pellets and feeding behavior was recorded. Then, the fish were killed with an overdose of MS-222.

2.4.6 In vivo evaluation of putative probiotics

Initially, separate groups of 20 rainbow trout were fed to satiation for 14 days with the experimental diet supplemented with putative probiotics at a concentration of ~5 × 10^7 cells g^{-1} or control diet. Then, fish were challenged i.p. with 0.1 mL $fish^{-1}$ of *V. anguillarum* or *V. ordalii* suspensions containing 3×10^5 and 5×10^4 cells mL^{-1}, respectively. These cell numbers led to the death of >80% of the fish populations as determined before (Section 2.3).The groups of control and treated fish were observed daily for 2 weeks, and the relative percent survival (RPS[1]) was calculated after Amend (1981). Challenge experiments were maintained as before (see Section 2.4.3). From this preliminary work, two isolates, i.e. SM1 and SM2 which were recovered from the gut of rainbow trout, were determined to be potentially beneficial, and were evaluated further using groups of 60 rainbow trout. The fish were fed with SM1 and SM2 supplemented diets containing ~10^8 and ~10^7 cells g^{-1} of feed, respectively, or with control diets three times daily for 14 days, and subdivided into groups of 25, before challenge, as above. The cell numbers of probiotics used in feed were verified as effective feeding dose (see Section 2.6). Additional sub-groups of 10 fish were used for immunological assays involving blood, serum or head kidney (HK) macrophages.

$$^{1}\text{RPS (\%)} = \left[1 - \left(\frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}}\right)\right] \times 100$$

2.4.7 Characterization of the bacterial isolates

2.4.7.1 Micro-morphology

Overnight cultures on TNA plates were used to study micro-morphology. Thus, a cell suspension in saline was spread on a sterile slide with a sterile loop (= smear) and subsequently stained by Hucker's modification of the Gram-stain (Hucker and Conn 1923), and examined at ×1000 magnification on a Kyowa light microscope. Also, colonies on the plates were examined visually to determine morphology and colour. The cell shape and colour, cell arrangement and staining reactions were noted.

2.4.7.2 Motility

Motility test was assessed microscopically from wet preparations. Thus, isolates were grown for 4 h at 26°C in TNB on a shaker. Then, 5 µL of the liquid culture was placed on a sterile microscope slide, a coverslip placed on top, with observation on a Kyowa light microscope, at a magnification of ×400, as before. Water movement and Brownian motion were taken into consideration when observing motility.

In a parallel experiment, motility was recorded by the development of diffuse growth extending outward from a stab mark made in semi-solid growth medium (after Karlsen *et al.* 2008). Thus, bacteria were cultured in semi-solid TNB containing 0.25% bacteriological agar (Agar no. 1; Oxoid). The bacterial cells from overnight cultures were inoculated, using a sterile needle, into the medium, which was incubated at 26°C, and motility monitored for 7 days by measuring the diameter of swimming zones.

2.4.7.3 Oxidase and catalase production

A piece of Whatman filter paper (No. 1) was moistened with 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich), which was streaked by touching a single colony grown overnight on TNA plates. A positive reaction for oxidase was recorded visually by the development of a purple colour within 30 seconds. Catalase production was indicative of rapid evolution of bubbles due to molecular oxygen being released when a drop of 5% H_2O_2 solution was added to a bacterial colony on a glass slide.

2.4.7.4 Optimum temperature and salinities for growth

In order to determine the salinity and temperature tolerances of the isolates, 190 µL volumes of medium containing different concentrations of NaCl [= TSB supplemented with 0, 2, 4, 6, 8, 10, 15 and 20% (w/v) NaCl] were inoculated with 10 µL of overnight bacterial suspensions [= ~10^9 cfu mL^{-1} in saline] in microtitre plates (Nalge Nunc), and incubated at 4, 15, 26, 37 and 45°C for up to 48 h. At 12, 24, 36 and 48 h, growth was measured at OD_{620} in a microplate absorbance reader using appropriate blank (= bacterial growth at 0 h) after thoroughly mixing the cultures.

2.4.7.5 pH growth tolerance

The pH (2–11) growth tolerance of the isolates was tested by growing in suitably modified TNB. Cultures (10 mL) at pH 2 to 11, with increments of pH 1 [adjusted with (v/v) 0.1 N NaOH or 0.1 N HCl] were grown at 26°C on a shaker (4 × *g*). The cultures were sampled after 12, 24, 36 and 48 h, and growth was determined as described in Section 2.4.7.4.

2.4.7.6 API 20E and API 20NE rapid identification systems

Isolates were examined using substrate utilization tests of API 20E for the identification of Enterobacteriaceae and other Gram-negative bacilli/rods, and API 20NE for non-fastidious and non-enteric Gram-negative rods following the manufacturer's instructions (Bio-Mérieux, Basingstoke, Great Britain). Thus, an overnight TNA culture was used to prepare the bacterial inocula in sterile saline. The inoculum was distributed into test strips, which were incubated at room temperature and read at 24 and 48 h. Results were read as positive or negative, scored against the reading table in the instruction sheet (= interpretive color chart), and the numerical profiles entered into the manufacturers' computer-based identification system to obtain the species identification.

2.4.7.7 Enzymatic profile

To identify the enzymatic profile by API ZYM enzyme substrate test (Bio-Mérieux), colonies (= a loopful) from TNA plates were suspended in sterile saline, inoculated onto the test strips and incubated for 4 h at 37°C, as recommended by the manufacturer. ZYM A and B reagents were added, and the substrate reactions were allowed to react for 5 min before recording in comparison with the manufacturer's guide.

2.4.7.8 Resistance to pepsin and pancreatin

Essentially the modified methods of Hosseini *et al* (2009) were used. Thus, cells from overnight TSB cultures were collected by centrifugation (2,500 × *g*, 30 min, 4°C), washed and resuspended in PBS (~10^9 cfu mL^{-1}). Then, 10 µL of this suspension was inoculated into 190 µL of TSB (pH 8.0) containing 0 and 1 mg mL^{-1} of pancreatin (Sigma-Aldrich), or into TNB (pH 2.0 and 3.0) supplemented with 0 and 3 mg mL^{-1} of pepsin (Sigma-Aldrich), respectively, in microtitre plates (Nalge Nunc). Resistance of the isolates to each of the enzymes was assessed by determining the bacterial growth at

OD_{620} after incubation at 37°C for 3, 4 and 5 h (pepsin) or at 37°C for 4, 5 and 6 h (pancreatin). Growth at 0 h was used as the blank.

2.4.7.9 Production of siderophores

Micro-organisms produce siderophores, which are recognized as efficient mechanisms for iron acquisition from the host or iron-limited environments. The chrome azurol S (CAS) assay (Schwyn and Neilands 1987) was used to determine siderophores as described by Brunt *et al.* (2007). In short, 60.5 mg CAS (Sigma-Aldrich) was dissolved in 50 mL de-ionized water, and mixed with 10 mL iron (III) solution [1 mM $FeCl_3.6H_2O$ (Sigma-Aldrich) in 10 mL of 10 mM HCl (BDH)] in a volumetric flask with stirring. This Fe-CAS solution was added slowly to 72.9 mg hexadecyltrimethylammonium bromide (HDTMA; Sigma-Aldrich) dissolved in 40 mL water. The resultant dark blue liquid was autoclaved (121°C, 15 min^{-1}), and mixed with an autoclaved mixture of 15 g bacteriological agar and 30.24 g piperazine-1,4-*bis* (2-ethanesulphonic acid) (PIPES; Sigma-Aldrich) dissolved in 750 mL of water with 12 g of a solution of 50% (w/v) NaOH to bring the pH to 6.8. After cooling at 60°C, the HDTMA-Fe-CAS blue complex and agar with PIPES buffer were mixed slowly by pouring along the glass wall and agitated with enough care to avoid foaming. Then, overnight TNB cultures of SM1 and SM2 were spotted (100 µL) onto CAS agar plates and incubated (26^{o}C for 72 h). The CSA assay detects colour change of Fe-CAS complex from blue to orange after chelation of the bound iron by siderophores (Schwyn and Neilands 1987).

2.4.7.10 Sensitivity to antibiotics

Antibiograms were performed on TNA plates seeded with 200 µL (~10^9 cells mL^{-1}) of the test bacteria cultured in TNB. Thereafter, antibiotic sensitivity discs (M5 Mastring; Mast Diagnostics, Bootle, Great Britain) were aseptically placed onto the freshly prepared lawns with incubation at 26^{o}C for 48 h. Sensitivity was indicated by the presence of a clear zone (of ≥ 3 mm) of growth around the discs.

2.4.8 Identification of the probiotics

SM1 and SM2 were identified by 16S rDNA gene sequencing, as follows:

2.4.8.1 Isolation of bacterial DNA

A loopful of bacterial culture from a TSA plate was inoculated into 10 mL of TNB, incubated at 26°C for 18 h, and harvested by centrifugation (2,500 × *g*, 15 min) at 4°C. Then, bacterial cells were lysed, and the DNA was extracted using a DNeasy tissue kit (Qiagen, Crawley, Great Britain), following the manufacturer's protocol, i.e. isolation of total genomic DNA for animal tissues.

2.4.8.2 Agarose gel electrophoresis

The isolated DNA was checked for purity, using standard methods (Sambrook *et al.* 1989), i.e. the presence of single bands following electrophoresis in agarose gels. Briefly, 1% (w/v) agarose (Sigma-Aldrich), was dissolved in 40 mL of 1× Tris-acetate-ethylenediaminetetraacetate (EDTA) buffer [TAE; 50× TAE buffer = 40 mM (w/v) Tris base (Sigma-Aldrich), 20 mM (v/v) glacial acetic acid (Sigma-Aldrich), 1 mM (w/v) EDTA (Sigma-Aldrich), pH 8.0] by heating in a microwave oven until complete dissolution of the agarose. The solution was allowed to cool to 50°C before the addition of 0.4 µL of 10 mg mL^{-1} (w/v) ethidium bromide (EtBr; Sigma-Aldrich). The gel solution was thoroughly mixed and poured into a gel casting tray avoiding bubbles with integral comb (14 wells). The gel was allowed to solidify for 20 min before the addition of electrophoresis buffer to cover the top of the gel followed by the careful removal of the comb to reveal the sample wells. The electrophoresis tank was topped up with enough TAE buffer to cover the gel to a depth of ~1 mm. Then 5 µL of sample DNA was mixed with 2.5 µL of 6× gel-loading buffer [0.25% (w/v) bromophenol blue (Sigma-Aldrich), 0.25% (w/v) xylene cyanol (Sigma-Aldrich), 25% (v/v) glycerol (Sigma-Aldrich), 1% (w/v) sodium dodecyl sulphate (SDS; Sigma-Aldrich), 150 mM (w/v) EDTA (pH 8.0), dissolved in distilled water and stored at room temperature] on a parafilm sheet, before loading the sample solution into the wells. Six µL of marker (GeneRuler 1 kbp DNA ladders; MBI Fermentas, Germany) was loaded in one of the wells for a competitive molecular weight determination of the DNA. Electrophoresis was carried out for 1 h and 15 min at 60 V or until the tracking dye had travelled to the bottom of the agarose gel. Following electrophoresis, the DNA banding patterns on the agarose gel were immediately observed by an ultraviolet transilluminator (UV Products, Cambridge, Great Britain). Photographic records were taken immediately on a gel documentation unit (Amersham Biosciences).

2.4.8.3 PCR amplification

Partial sequencing of the 16S rDNA genes (>925 bp) of new isolates was carried out essentially as described by Orozova *et al.* (2009), after the 16S rDNA gene was amplified by PCR with oligonucleotide primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′). These are effective for most Gram-positive bacteria (Lane 1991). The amplification mixture (50 μL) was prepared in a sterile 0.2 mL PCR tube (Greiner, Stonehouse, Great Britain) with 30.7 μL of sterile Milli-Q water (Milli-Q purification system, Millipore, Watford, Great Britain), 10.0 μL of *Taq*-dNTPs buffer mix [i.e. 100 μL 10× PCR buffer (Biomix; Bioline, London, Great Britain); 2 μL each of 100 mM dATP, dTTP, dGTP and dCTP (Sigma-Aldrich); 92 μL Milli-Q water], 2.0 μL of 50 mM magnesium chloride ($MgCl_2$; Bioline), 2.5 μL (5 pmol μL^{-1}) of each primers 27f and 1492r (MWG Biotech, Ebersberg, Germany) and 2.0 μL of DNA template, and centrifuged for a few seconds. A control contained 2 μL of sterile Milli-Q water instead of DNA. PCR amplification was carried out on a Bio-Rad iCycler (version 3.021; Hercules). Template DNA was initially denatured at 95°C for 5 min and the machine paused for the addition of 0.3 μL *Taq* polymerase (BIOTAQ, Bioline), followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and an extra extension step at 72°C for 10 min, and a completion holding temperature of 4°C. The integrity of the PCR products was assessed by the development of single bands following gel electrophoresis as previously described, whilst ensuring that the control sample gave no banding patterns as a result of contamination.

2.4.8.4 Sequencing of PCR products

Prior to sequencing, the 16S rDNA fragment obtained from the PCR was subjected to polyethylene glycol precipitation (Embley 1991). Purified PCR products (2−10 μL depending on the brightness of the band) in duplicate microfuge tubes were dried in the vacuum-drier for 30 min and sent for DNA sequence analysis to MWG Biotech with sequencing primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 685r3 (5′-TCTRCGCATTYCACCGCTAC-3′) (10 pmol μL^{-1}; MWG Biotech). The derived nucleotide sequences were analyzed and aligned with the STADEN package (Staden 1996). The corrected 16S rDNA gene sequences were compared with those in the

GenBank databases (www.ncbi.nlm.nih.gov/blast) by using the BLASTN program (Version 2.2.12).

2.5 Survival of probiotics on feed

The viability of probiotics SM1 and SM2 in the feed was determined by means of the total viable counts on TNA plates following storage of the probiotic-supplemented diets (~10^8 cells g^{-1}) either at 4°C or room temperature for 1 month. For this, 1 g amounts of feed were homogenized in 9 mL volumes of saline (see Section 2.4.4) and 10-fold dilutions prepared to 10^{-6} in fresh saline, before 0.1 mL volumes were spread over TNA plates. Colony counts were determined after incubation at room temperature for 7 days (Brunt and Austin 2005).

2.6 Effective feeding dose of the probiotics

The effective dose of SM1 and SM2 was determined by using feeds containing 10^5, 10^6, 10^7, 10^8 and 10^9 cells g^{-1} feed. Groups of 20 rainbow trout were fed three times daily for 14 days and then challenged i.p. with *V. anguillarum* or *V. ordalii* and monitored for mortalities over 14 days (Brunt and Austin 2005), as before.

2.7 Effective feeding duration of probiotics

Studies with probiotics to date have employed different feeding durations, for example 1–8 weeks feeding regimes, leading to improved disease resistance in farmed fish (Robertson *et al.* 2000; Brunt *et al.* 2007; Aly *et al.* 2008; Pan *et al.* 2008; Son *et al.* 2009), but the basis for choosing these periods is often unclear. Therefore, optimal probiotic feeding duration for sustaining the maximum level of disease protection in rainbow trout against *Vibrio* infections was determined. Thus, groups of rainbow trout were fed three times a day for up to 4 weeks with diets supplemented with SM1 (~10^8 cells g^{-1}) or control diets. During this feeding regime, sub-groups of 20 fish were removed at weekly intervals to challenge i.p. with *V. anguillarum*, and mortalities were recorded daily over 2 weeks and the RPS was calculated, as before. Additional sub-groups of 10 fish were sampled weekly for immunological assays.

2.8 Synergistic effects of probiotics

The hypothesis about synergistic effects on health, i.e. improvement or prolongation of the desirable effects (Timmerman *et al.* 2004) from using mixtures of probiotic bacteria (= either multistrain or multispecies formulations that may complement each other) was investigated. Thus, groups of 60 rainbow trout were kept under feeding regimes either containing an equi-mixture of SM1 (5×10^7 cells g^{-1}) and SM2 (5×10^7 cells g^{-1}) or control diets three times daily for 14 days. Then, fish were challenged i.p. with *V. anguillarum* or *V. ordalii*, and monitored for mortalities over 14 days, and the RPS was calculated. As before, sub-groups of fish were used for immunological assays.

2.9 Long-term beneficial effects of probiotics

It is argued that the fish immune system lacks memory (see Ortuño *et al.* 2002), and as such the duration of probiotic induced beneficial responses, which are primarily mediated by innate immunity (Brunt and Austin 2005; Kim and Austin 2006a; Brunt *et al.* 2007) may inevitably be shorter than that of the specific immune responses. Therefore, the duration of disease protection in rainbow trout after feeding with probiotics was determined. Groups of rainbow trout were fed three times daily for 2 weeks with SM1 supplemented or control diets, as before. Then, fish were switched to standard commercial diet for up to 5 weeks. During the withdrawal period of dietary probiotics, two groups of 20 fish were removed at weekly intervals to challenge with *V. anguillarum* and an additional 10 fish were used for immunology. The groups of control and treated fish were observed daily for 2 weeks, and the RPS was calculated.

2.10 Efficacy of the cellular components of probiotics

The efficacy of sub-cellular components of the probiotics to confer host protection was evaluated. Thus, groups of 15 rainbow trout were inoculated i.p. with 0.1 mL volumes of 2.0 ± 0.5 mg mL^{-1} extracellular proteins (ECPs), cell wall proteins (CWPs) and whole cell proteins (WCPs) derived from SM1 and SM2, or with phosphate-buffered saline (PBS; Sigma-Aldrich) as control. Then, fish were maintained with control diet, before challenge with *V. anguillarum* and sampled for immunology on day 8. As before, mortalities were recorded daily over 14 days and the RPS was calculated.

Before inoculation into fish, the concentration of total protein present in the cellular components, which were dissolved in PBS (see Section 2.11), were measured with BioAssay Systems (Hayward, CA, USA) QuantiChrom™ protein assay kit (QCPR-500). Thus, standard [Bovine serum albumin (BSA)] and samples were diluted in PBS according to the manufacturer's instructions. An aliquot (10 µL) of diluted standard and samples were transferred into wells of flat bottom 96-well plates (Nalge Nunc, Loughborough, Great Britain). Then, 200 µL of working reagent, which was supplied with the kit, was added to each well and mixed gently. The intensity of colour obtained was measured at OD_{620} in a microplate absorbance reader (Sunrise; Tecan, Reading, Great Britain). The OD value of a blank was deducted from the OD of standard, and plotted against the protein concentrations of standard to produce the standard curve. Then, the OD values of the samples were plotted onto the standard curve to obtain the protein concentration in the sample. If necessary, the proteins were re-diluted in PBS to achieve the required concentration (= 2 mg mL^{-1}) and stored at −20°C for subsequent use.

2.11 Preparation of sub-cellular proteins

2.11.1 Upscaling of probiotic cultures

A loopful of SM1 or SM2 from a TNA plate was inoculated into 10 mL of TNB and incubated overnight at 26°C. Then, cultures were inoculated at a 1:100 dilution in TNB and incubated (18 h, 26°C) on a shaker at 4 × *g*. These cultures were used to prepare the sub-cellular proteins.

2.11.2 Collection of extracellular proteins (ECPs)

ECPs were separated essentially as described by Barbey *et al.* (2009) with slight modifications. Briefly, bacterial cells were removed by centrifuging at 20,000 × *g* for 30 min at 4°C (Avanti J-26 XP centrifuge; Beckman Coulter, Brea, CA, USA) and the supernatants were filtered through a 0.22 µm porosity filter (Millex-GS; Millipore, Cork, Ireland). Then a final concentration of 10% (w/v) trichloroacetic acid (TCA; Sigma-Aldrich) was added to the supernatant, mixed well (1 min vortex) and placed in an ice bath for 3 h. The mixture was transferred to a 1.5 mL capacity Eppendorf tube, the precipitated proteins were harvested by centrifugation (20,000 × *g*, 30 min) at 4°C using a microcentrifuge (Microfuge 22R centrifuge; Beckman Coulter), the pellet was

washed four times with 1 mL of cold methanol (BDH), and dried in a ~95°C heat block for 5–10 min to drive off the residual methanol. Finally, the pellet was washed twice with PBS and redissolved in PBS.

2.11.3 Separation of cell wall proteins (CWPs)

The modified method of Abbass *et al.* (2010) was used. Thus, suitably upscaled bacterial cultures were centrifuged (2,200 × *g*, 15 min, 4°C) in a Mark IV refrigerated centrifuge (Baird Tatlock), and the cell pellets were resuspended in 0.5 mM NaCl and washed twice with 0.05 M Tris-HCl buffer (Sigma-Aldrich), pH 7.8. Then, the cells were resuspended to 20 mL with 0.05 M Tris-HCl (pH 7.8) containing protease inhibitor, 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), and disrupted by sonication (6 × 5 min; after each 5 min sonication the sample was incubated for 4 min in ice) on ice with a sonicator (MSE ultrasonic power unit; MSE, London, Great Britain). Cell disruption was checked by microscopy. The sonicated product was centrifuged (2,200 × *g*, 5 min, 4°C) to remove cell debris and the cell-walls were separated by centrifugation (20,000 × *g*, 30 min) of the supernatant at 4°C. The pellet was resuspended in 100 mM NaCl, washed twice in PBS and suspended in the same buffer.

2.11.4 Preparation of whole cell proteins (WCPs)

The WCPs were prepared according to Abbass *et al.* (2010) with slight modification. The cells were collected by centrifugation (2,200 × *g*, 15 min, 4°C), the cell pellets were collected and resuspended with 0.5 mM NaCl, washed twice with Milli-Q water, then resuspended in Milli-Q water containing 1mM PMSF and frozen at −20°C. Cells in suspension were thawed and disrupted by sonication on ice for 6 × 5 min, mixed with equal volumes (v/v) of lysis buffer [4 g SDS, 20 mL glycerol, 10 mL β-mercaptoethanol (Sigma-Aldrich) and 12.5 mL 0.5 M Tris-HCl (pH 6.8) per 100 mL of Milli-Q water] and kept on ice for 30 min. The supernatant was collected following centrifugation and contains the WCPs. Precipitation of proteins was done following the methanol-chloroform method (Wessel and Flugge 1984). Briefly, 0.4 mL methanol (BDH) was added to 0.1 mL lysate, vortexed well, then 0.1 mL chloroform (BDH) was added, and vortexed again before addition of 0.3 mL distilled water. The mixture was vortexed and spun for 2 min at 8,950 × *g* at 4°C. The top aqueous layer was carefully

removed and 0.3 mL of methanol added, vortexed and centrifuged (8,950 × *g*, 5 min, 4°C) to pellet the proteins. The protein pellet was air dried and suspended in PBS.

2.11.5 Electrophoresis

One-dimensional denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of soluble protein fractions in ECPs, CWPs and WCPs extracts was carried out according to Laemmli (1970). Briefly, concentrated proteins (~1 mg mL^{-1}) were measured as before (see Section 2.10), mixed 1:1 with 2× Laemmli sample buffer [2.5 mL 0.5 M Tris-HCl (pH 6.8), 2 mL glycerol, 4 mL 10% SDS, 0.31 g dithiothreitol (DTT, Sigma-Aldrich), 0.04% bromophenol blue; make up to 10 mL with distilled water], boiled at ~100°C for 10 min in a heating block, and loaded (10–30 µL protein sample $well^{-1}$) into Tris-HCl-SDS gels with 4% (w/v) polyacrylamide stacking, and 10% (for ECPs and WCPs) or 12% (w/v) polyacrylamide separating (= resolving) gels. Also, 10 µL of prestained molecular-mass standards (Bio-Rad, Hemel Hempstead, Great Britain) were loaded in one lane on all gels. The resolving gel solutions (20 mL) contained 70 µL of 10% ammonium peroxodisulphate (APS; Sigma-Aldrich) and 15 µL of N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma-Aldrich), whereas stacking gels (10 mL) contained 50 µL 10% APS and 10 µL TEMED. Electrophoresis was carried out in a Mini Protean II electrophoresis chamber (Bio-Rad) for ~1.5 h at 150 V constant voltage, in running buffer [12.0 g Tris, 57.6 g glycine (Sigma-Aldrich), 2.0 g SDS; made up to 2.0 L with distilled water] at room temperature. After the electrophoretic separation, protein bands were visualized by staining the gel for 1 h with brilliant blue G solution (Sigma-Aldrich) followed by destaining in methanol-acetic acid-water solution (40:10:50) for 3 h. Densitometry of gels was performed with the aim of assigning relative molecular masses to the ECPs, CWPs and WCPs separated bands. Protein bands were digitally imaged using a Canon CanoScan 3000F scanner (Canon, Lake Success, NY, USA).

2.12 Innate immune response

Immunological assays (5 or 10 fish $group^{-1}$) were involved with blood, serum or head kidney (HK) macrophages. Prior to sampling, fish were anesthetized with 0.01% MS-222 and individual fish were sampled once to avoid multiple bleeding and/or handling stress, which may lead to an influence on the assays.

2.12.1 Samples for immunology

Blood was collected by venepuncture using syringes coated with heparin (Sigma-Aldrich) and transferred immediately into 9 mL capacity lithium heparin vacuettes (Greiner) on ice. For serum, the blood was transferred into vacuettes containing Z Serum Clot Activator (Greiner) and allowed to clot at 4°C for 4 h. The sera were separated by centrifugation (2,000 × *g* for 25 min at 4°C) and stored at −70°C until required.

Head kidney (HK) macrophages were isolated largely according to the method of Secombes (1990). Thus using aseptic techniques, the anterior HK was removed, placed into a glass homogenizer (30 mL capacity; VWR-Jencons, East Grinstead, Great Britain) containing 5 mL of L-15 medium (Sigma-Aldrich), crushed and passed through a 100-μm nylon mesh into a Petri dish. The mesh was rinsed with additional 1 mL quantities of L-15 medium. The resulting cell suspension was slowly layered onto a 51% Percoll cushion [51 mL Percoll (Sigma-Aldrich), 10 mL 10% Hank's balanced salt solution (HBSS; Sigma-Aldrich), 39 mL sterile distilled water] and centrifuged at 400 × *g* for 30 min at 4°C. The band of cells lying at the medium/Percoll cushion interface was collected with a Pasteur pipette and washed twice with L-15 medium by centrifugation at 800 × *g* for 10 min. The number of cells was adjusted to ~10^6 cells per mL (by microscopy and use of a haemocytometer slide) in fresh L-15 medium supplemented with 0.1% (v/v) foetal bovine serum (FBS; Sigma-Aldrich) and 10 μL mL^{-1} of heparin (Sigma-Aldrich). Cell viability was determined at ×400 magnification of preparations containing equal mixtures of the cell suspension and 0.4% (w/v) aqueous trypan blue solution (Sigma-Aldrich). Dead cells developed an intracellular blue coloration within 5 min.

2.12.2 Cellular immune parameters

2.12.2.1 Macrophage phagocytosis

Phagocytic activity of HK macrophages was determined spectrophotometrically involving the measurement of stained yeast cells that had been phagocytosed (internalization of yeast) by cells (Seeley *et al.* 1990). Briefly, 1 mL of macrophage suspension (~10^6 cells mL^{-1}) was mixed with 2 mL of Congo red-stained yeast cells (yeast cell: macrophage ratio of 30:1) in microfuge tubes. Following incubation at room

temperature for 1 h, 1 mL ice-cold HBSS was added and 1 mL of histopaque (density of 1.077 g mL^{-1}, Sigma-Aldrich) was injected by a syringe into the bottom. The samples were centrifuged at 850 × *g* for 5 min to separate macrophages from free yeast cells. Macrophages located at the medium/histopaque interface were harvested with a Pasteur pipette and washed twice in HBSS. The cells were then resuspended in 1 mL trypsin-EDTA solution (5 g L^{-1} trypsin and 2 g L^{-1} EDTA, Sigma-Aldrich) and incubated at 37°C overnight. The OD of the samples was measured at 510 nm with a spectrophotometer (Spectronic Genesys 20, Thermo Electron Corporation, Town, Country) using trypsin-EDTA as a blank.

Yeast cells (*Saccharomyces cerevisiae*) were prepared as described previously (Walsh and Luer 1998) by staining 1 g dry yeast (NT 116, Anchor Bio-Technologies, Cape Town, South Africa) with 3 mL of 0.87% (w/v) Congo red (Sigma-Aldrich) in PBS, mixed thoroughly and incubated for 15 min at room temperature. Then 7 mL of PBS was added to the mixture, vortexed, and inactivated at 121°C 15 min^{-1}. These cells were washed three times in HBSS, the concentration adjusted to ~3 × 10^7 cells mL^{-1} and stored at 4°C until use.

2.12.2.2 Respiratory burst activity

Intracellular respiratory burst activity of HK macrophages was detected from the reduction of nitroblue tetrazolium (NBT; Sigma-Aldrich) to formazan as a measure of superoxide anion (O_2^-) production (Secombes 1990). Thus, 100 µL of macrophages was pipetted into the wells of 'U' bottom microtitre plates (Nalge Nunc) and centrifuged at 700 × *g* for 20 min. The supernatant was removed, and the wells were washed with L-15 medium. Then, 100 µL of NBT (1 mg mL^{-1} in L-15 medium) and 100 µL of phorbol 12-myristate 13-acetate (Sigma-Aldrich) dissolved at 1 µg mL^{-1} in dimethyl sulphoxide (DMSO; Sigma-Aldrich) was added with incubation for an hour at room temperature. The medium was removed, and the cells were fixed with 100% (v/v) methanol for 2–3 min and again washed twice with 70% (v/v) methanol. The plate was then air dried at room temperature before 120 µL of 2 M potassium hydroxide (KOH; Sigma-Aldrich) and 140 µL of DMSO were added to each well to dissolve the resulting formazan blue crystals. The OD was read in a microplate reader at 620 nm against a KOH/DMSO blank.

Parallel experiments using whole blood were also carried out to evaluate the respiratory burst activity as described previously (Pieters *et al.* 2008). Thus, 50 µL of blood was pipetted into the wells of 'U' bottom microtitre plates (Nalge Nunc) and incubated for 1 h at room temperature to facilitate adhesion of the cells. The supernatant was gently removed and the adhered cells were washed three times with PBS. After washing, 50 µL of 0.2% (w/v) NBT in PBS was added to the wells and incubated for an hour at room temperature. The supernatant was removed, and the cells were fixed with 100% (v/v) methanol for 3 min, and then washed three times with 30% (v/v) methanol. The plates were air-dried before 60 µL of 2 M KOH and 70 µL DMSO were added to each well to dissolve the formazan blue crystals. The OD of the resulting solution was read in a microplate reader at 550 nm against a KOH/DMSO blank.

2.12.2.3 Macrophage peroxidase

The total peroxidase content present inside HK macrophages was measured as described previously (Salinas *et al.* 2008). In brief, ~10^6 cells mL^{-1} macrophage suspension were lysed with 0.002% cetyl trimethylammonium bromide (CTAB; Sigma-Aldrich) for 5 min in microfuge tubes. After centrifugation (400 × *g*, 10 min), 150 µL of the supernatants were transferred to a 96-well microtitre plate (Nalge Nunc) containing freshly prepared peroxidase substrate, i.e. 25 µL of 10 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma-Aldrich) and 25 µL of 5 mM H_2O_2 (Sigma-Aldrich). The colour-developing reaction was stopped after 2 min by adding 50 µL of 2 M sulphuric acid (H_2SO_4; BDH) and the OD was read at 450 nm in a microplate absorbance reader. Blank samples without macrophages were also analysed.

2.12.2.4 Bacteriocidal activity

The bacteriocidal activity of HK macrophages was examined using the method of Secombes (1990). Briefly, 100 µL of HK cell suspension (~10^6 cells mL^{-1}) was added to 'U' bottom 96-well microtitre plates and centrifuged at 700 × *g* for 20 min. The supernatant was removed, the macrophage monolayer washed off with L-15 and resuspended with 100 µL L-15 containing 5% FBS. Overnight cultures of *V. anguillarum* and *V. ordalii* in TNB were centrifuged, and the cell pellet washed and suspended in saline, before adjusting to ~10^6 cells mL^{-1} using counts obtained with a haemocytometer. Then, 20 µL of the bacterial suspension was added to triplicate wells containing macrophages or medium only and centrifuged at 150 × *g* for 5 min to bring

the bacteria into contact with the cells. The plate was incubated at 22°C for 0 and 5 h to allow killing activity. At the end of the incubation period, the killing was stopped by lysing the macrophages with the addition of 50 µL Tween-20 [0.2% (v/v) in distilled water] per well. Then 100 µL of TNB was added and surviving bacteria allowed to grow overnight at 22°C. The amount of bacteria existing in the wells was determined by addition of 10 µL 5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich), shaking the plate and reading the OD at 620 nm 15 min later. The data were converted to a killing index (KI): KI = OD $Time_{5h}$/OD $Time_{0h}$. The lower the index, the more bacteria had been killed.

2.12.2.5 Number of erythrocytes and leucocytes

Unclotted blood was used to determine the number of erythrocytes and leucocytes using flow cytometry after Newaj-Fyzul *et al.* (2007). Thus, a staining solution 3,3'-dihexyloxacarbocyanine [DiOC6(3); Sigma-Aldrich] was prepared in absolute ethanol to 500 µg mL^{-1} and held in the dark. This stock solution was diluted tenfold in HBSS immediately prior to use. Then, 5 µL of freshly obtained blood was added to triplicate test tubes, each containing 790 µL of HBSS, 5 µL of DiOC6(3) dye solution and 5 µL of heparin. These were mixed gently and incubated at room temperature for 2 min. Blood cells were analysed with a flow cytometer (Cyflow SL; Partec, Münster, Germany). Forward scatter (FSC), side scatter (SSC) and green fluorescence (FL-1) of each cell was measured. All data were acquired using the Flomax (Partec) software package and analysed with WINMDI 2.8 (Windows Multiple Document Interface for Flow Cytometry: J. Trotter).

2.12.2.6 Leukocrit value

In a parallel experiment, responses for white blood cells were studied by measurement of the leukocrit value. Thus, blood was drawn into heparinized microhaematocrit tubes (1.15 × 1.15 × 75 mm; Hawksley, Lancing, Great Britain) by capillary action, the ends of the tubes sealed and centrifuged in a haematocrit centrifuge (MSE, London, Great Britain) for 5 min at 12,000 × *g*. The leukocyte layer, i.e. the whitish buffy coat above packed red cells, was measured, and the leukocrit value was defined as the percentage of the leukocyte layer height against total blood volume inside the tube. The measurements were performed on a microhaematocrit reader (MSE).

2.12.3 Humoral immune parameters

2.12.3.1 Serum lysozyme

A turbidometric assay using lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich) was followed to determine the lysozyme activity in serum as described by Ellis (1990). Thus, 2 mL of *M. lysodeikticus* at a concentration of 0.2 mg mL^{-1} (w/v) in 0.05 M sodium phosphate buffer, pH 6.2 (SPB; Sigma-Aldrich) was added to 100 µL of serum sample. As a negative control, SPB replaced serum. The decrease in OD was recorded at 530 nm after 1 and 5 min at 22°C. A unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001 units min^{-1}.

2.12.3.2 Alternative complement activity

The activity of the serum alternative complement pathway (ACH_{50}) was examined by adapting the method of Giclas (1994) and using sheep red blood cells (SRBC; Oxoid) as targets. All reagents, cells and sera were kept on ice throughout this protocol unless otherwise mentioned. Briefly, SRBC in Alsevier's solution were centrifuged at 400 × *g* for 10 min, pelleted and washed twice with ethyleneglycoltetraacetic acid (EGTA)-Mg^{2+}-gelatin/veronal buffer (GVB) [0.01 M EGTA (Sigma-Aldrich) and 0.01 M $MgCl_2.6H_2O$ (Sigma-Aldrich) in gelatin/veronal-buffered saline (0.142 M NaCl, 0.01 M sodium 5,5'-diethyl barbiturate and 0.1% gelatin)] and finally resuspended in the same buffer to a concentration of ~4×10^8 cells ml^{-1}. Seven serial 2-fold dilutions of test and control sera were prepared in ice-cold EGTA-Mg^{2+}-GVB buffer, mixed well after each separate dilution step, before 100 µL of each dilution was transferred to duplicate tubes. In separate tubes, 100 µL EGTA-Mg^{2+}-GVB buffer and 100 µL of distilled water were used as the background lysis and total lysis control. Then, 50 µL of SRBC suspension was added to each tube and incubated for 60 min at 21°C with occasional shaking to keep the cells in suspension. The haemolytic reaction was stopped by adding 1.2 mL of ice-cold 0.15 M NaCl (w/v) to each tube. The mixtures were centrifuged at 1,250 × *g* for 8 min at 4°C to spin down the remaining SRBC. The OD_{412} of each supernatant was measured after zeroing the spectrophotometer with water or EGTA-Mg^{2+}-GVB. The degree of haemolysis (*y*), defined as the fraction of cells lysed, was calculated taking OD_{412} value for a given tube divided by the OD_{412} of the total lysis tube. On two-cycle log-log paper, the reciprocal of the serum dilution on the *y* axis against $y/(1 - y)$ on the *x* axis was plotted. Values of *y* in the range of 0.1–0.9 were used for the calculation. The

best straight line between the points was drawn and the ACH_{50} read from the graph as the reciprocal of the dilution where $y/(1 - y) = 1$, corresponds to lysis of 50% of the cells (Kabat and Mayer plot: Mayer 1961).

2.12.3.3 Serum antiproteases

Total antiprotease activity was determined as indicated by the capacity of serum to inhibit trypsin activity (Ellis 1990; Magnadóttir *et al.* 1999). Briefly, 20 µL of serum was incubated with 20 µL of standard trypsin solution (1000–2000 BAEE, 5 mg mL^{-1}; Sigma-Aldrich T-7409) for 10 min at 22°C in Eppendorf tubes. Then, 200 µL of 0.1 M PBS (pH 7.0) and 250 µL of 2% (w/v) azocasein (Sigma-Aldrich) in PBS were added, and incubated for 1 h at 22°C. The reaction was stopped by the addition of 500 µL of 10% (v/v) TCA, incubated for 30 min at 22°C, and then centrifuged at 6000 × *g* for 5 min. The supernatants (100 µL) were transferred to a 96-well microtitre plate (Nalge Nunc) containing 100 µL $well^{-1}$ of 1 N sodium hydroxide (NaOH, BDH). The OD was read at 450 nm using a plate reader. For a positive (100%) control, buffer replaced the serum, and for a negative control, buffer replaced both serum and trypsin. The inhibitory ability of antiprotease was expressed in terms of percentage trypsin inhibition (Zuo and Woo 1997).

$$\%\ \text{Trypsin inhibition} = \frac{\text{Trypsin OD} - \text{Sample OD}}{\text{Trypsin OD}} \times 100$$

2.12.3.4 Serum bacterial killing

The serum bacterial killing was performed according to Villamil *et al.* (2003), which was measured by comparing the growth of *V. anguillarum* in serum with that observed in TNB. Colonies of pathogen from TNA were grown in TNB for 24 h at 26°C. The culture was centrifuged at 2,500 × *g* for 20 min at 4°C, the cell pellet washed twice and resuspended in 0.9% (w/v) saline, and the concentration adjusted to ~10^8 cells mL^{-1}, as before. For the assay, 33 µL of serum samples or TNB (control) were placed in wells of a 96-well microtitre plate. Then, 100 µL of bacterial suspension was added to each well, mixed with micropipette and incubated for 5 h at room temperature. The plate was centrifuged (10 min at 200 × *g*) in a Sigma 4K15 centrifuge (Newport Pagnell, Great Britain), the supernatant discharged and 100 µL of 0.5 mg mL^{-1} MTT was added to the wells. After 15 min of incubation in the dark, the plate was read at an OD of 620 nm

and the percentage of surviving bacteria was calculated [(OD_{620} of each sample ÷ OD_{620} of control *V. anguillarum*) × 100].

2.12.3.5 Serum peroxidase

The total peroxidase content present in serum was calculated according to the reported method (Díaz-Rosales *et al.* 2006). Briefly, 15 µL of serum was diluted with 35 µL of Ca^{2+}- and Mg^{2+}-free HBSS (Sigma-Aldrich) in flat-bottomed 96-well microtitre plates (Nalge Nunc). Then, freshly prepared peroxidase substrate, i.e. 50 µL of 20 mM TMB and 50 µL of 5 mM H_2O_2 were added. Subsequently, the serum mixture (150 µL) was transferred from each well to new 96-well microtitre plates. The colour-developing reaction was stopped after 2 min by adding 50 µL of 2 M H_2SO_4 (BDH) and the OD was read at 550 nm in a microplate absorbance reader. Blank samples without serum were also recorded.

2.12.3.6 Serum total immunoglobulin (Ig)

The method of Siwicki and Anderson (1993) as described by Panigrahi *et al.* (2005) was followed to determine the total immunoglobulin level in serum. Thus, 100 µL of serum sample (100-fold dilutions in PBS) was mixed with an equal volume of 12% (v/v) solution of polyethylene glycol (10,000 MW, PEG; Sigma-Aldrich) and incubated for 2 h at room temperature that helped in bringing down the Ig molecules. The Ig molecules were removed by centrifugation (5,000 × *g*, 4°C) and the protein content was determined as mentioned for total protein determination by the Bradford method (Section 2.15.1). This value was subtracted from the total protein level, which corresponds to the total Ig content and was expressed in mg mL^{-1}.

2.13 Growth performance of fish

After the dietary intake of SM1 and SM2 supplemented feeds for 14 days, groups of 25 fish were used to determine growth performance in terms of percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), hepato-somatic index (HSI) and spleen mass index (SMI). Uneaten feed pellets were collected from the tank bottom 30 min after each feeding and counted to estimate feed/protein intake.

Weight gain (%) = $[(W_t - W_0) \div W_0] \times 100$

SGR (% day^{-1}) = $[(\ln W_t - \ln W_0) \div (t - t_0)] \times 100$

where, W_0 is the initial individual weight of fish

W_t is the individual weight at time t

t represents time in days

FCR = Total feed consumed (g) ÷ Total wet weight gain of animal (g)

Protein intake (PI) = [Feed intake (g) × percent protein in the diet]

PER = [Total wet weight gain of animal (g) ÷ Total protein intake (g)]

HSI = [Weight of liver (g) ÷ Weight of whole fish (g)] × 100

SMI = [Weight of spleen (g) ÷ Weight of whole fish (g)] × 100

2.14 Influence on digestive enzymes

2.14.1 Crude enzyme sample

After 14 days feeding with isolates SM1 and SM2, sub-groups of 10 fish were killed with an overdose of anesthetic 2 h after their last meal and dissected by keeping on ice plate. Then pyloric caeca, stomach and intestine with digesta in each fish were dissected immediately, and pooled tissues were homogenized in 20 volumes (v/w) of ice-cold PBS using a glass tissue grinder (30 mL capacity; VWR-Jencons) and then subjected to an ultrasonic cell disintegrator (Sonicator; MSE). The homogenates were centrifuged (10,000 × *g*, 30 min, 4°C) and the resultant clear supernatants were adjusted (or if needed diluted further) to a protein concentration of 1 mg mL^{-1} (after Bradford's method) and stored at −20°C until used for enzyme assay. Many previous studies used empty intestine where the enzymes localized in mucus may be assayed. However, samples from digestive organs with digesta may contain a lot of diverted enzymes of the stomach, pancreas and intestine, which might be involved in a complex network to complete hydrolysis of ingested nutrients. Thus, the activities of the digestive enzymes in these samples may hold information more of the useful ones reflecting actual physiological conditions (Li *et al.* 2009).

2.14.2 API ZYM assay

Sixty five µL of supernatants were inoculated into each well of API enzyme substrate (Bio-Mérieux) and incubated for 4 h at 37°C. The reaction was determined from the colour development following incubation for 5–10 min after addition of 1 drop each of

reagents ZYM A and ZYM B at room temperature. The resulting colours were estimated under natural light and scored as 0–5, according to a colour scale supplied by the manufacturer (Bio-Mérieux).

2.15 Changes in biochemical parameters

2.15.1 Serum total protein, albumin and globulin

The Bradford method was used for total protein assay (after Simonian and Smith 2006). For this, 100 µL of serum sample (100-fold dilutions in PBS) was placed into Eppendorf tubes, mixed with 1 mL of Bradford reagent (Sigma-Aldrich), vortexed and incubated for 2 min at room temperature. The OD was read at 595 nm using a 1 cm path length microcuvette (Fischerbrand, Loughborough, Great Britain). Separate tubes containing 100 µL of PBS and 1 mL of Bradford reagent served as blanks. Then, the OD of the unknown protein sample was plotted on a standard curve to calculate the protein concentration. The standard curve was obtained by plotting the OD against known protein concentrations ranging from 0.25 to 1.0 mg mL^{-1} of BSA in PBS. The total protein content in serum was also determined as described in Section 2.10 using a commercial kit.

Albumin was measured by the bromocresol green binding method using BCP albumin assay kit (DIAP-250; BioAssay Systems) according to the manufacturer's instruction. The intensity of colour obtained with the kit was measured at 620 nm, being directly proportional to the albumin concentration in the sample. The globulin content was calculated by subtracting albumin values from total serum protein.

2.15.2 Serum haemoglobin (Hb), urea, creatinine and glucose

Commercially available kits were used to determine the quantitative colorimetric concentrations of serum hemoglobin (DIHB-250, QuantiChrom™ hemoglobin assay; BioAssay Systems), urea (DIUR-500, QuantiChrom™ urea assay; BioAssay Systems) and creatinine (DICT-500, QuantiChrom™ creatinine assay; BioAssay Systems), respectively. The serum glucose concentration was measured enzymatically using the Glucose Hexokinase Reagent Set™ method (Pointe Scientific).

2.16 Statistics

Each experiment was replicated three times within the experiment and the complete experiment was performed in triplicate, unless otherwise specified. The results were presented as mean ± SD (standard deviation of the mean), and were statistically analysed by one-way analysis of variance (ANOVA) and Duncan's comparison of means when necessary. Percentage data and ratio values were transformed to square-root arcsine values to homogenize variance. All statistical tests were conducted using the computerized software Statistical Package for Social Sciences (SPSS; Release 14.0, SPSS, Chicago, IL, USA). Differences were considered statistically significant when $P < 0.05$. The data were plotted using the program Microsoft Excel (Microsoft Co., Seattle WA).

CHAPTER 3 – RESULTS

3.1 The development of probiotics

3.1.1 Isolation and selection of candidate probionts

The intestinal content of rainbow trout and Atlantic salmon were used as a source of potential probiotics, with the recovery of average viable bacterial counts of 5.4 ± 0.8 × 10^6 from 1.0 g quantities of the intestinal contents on TSA. The majority of the colonies were milky white/cream, smooth and round, although a comparatively few were yellow and orange in colour. Ninety representatives of the various colony types were examined for antagonistic activity against *V. anguillarum* and *V. ordalii* by cross streaking.

Table 3.1 The inhibitory properties and effects of the bacterial isolates on rainbow trout.

Isolate reference no.	Inhibitory activity to:		No. of mortalities or sign of disease in fish following i.p./i.m. injection	
	V. anguillarum	*V. ordalii*	i.p. (n = 5)	i.m. (n = 5)
SL2	+	+/−	4	4
SL6	+	+	0	0
SL7	+	+	0	0
SL12	+	+	0	0
RT2	+	+	0	0
SM1	+	+	0	0
RT6	+	+/−	2	4
RT7	+	+	4	3
RT8	+	+	3	2
RT12	+	+	0	0
RT16	+	+	0	0
RT20	+	+	2	4
RT25	+	+	4	3
SM2	+	+/−	0	0
RT29	+	+	0	0
SM3	+	+	0	0
SM4	+/−	+	4	0

+ (overgrowth); +/− (slight overgrowth)

Initially, 17 out of 90 bacterial isolates (= ~19% of the total) were selected due to their inhibitory properties, either by overgrowing or interrupting the growth of pathogens (Table 3.1). The putative probionts were then assessed for the lack of pathogenicity in rainbow trout following challenge via the i.p. or i.m. routes with 0.1 mL suspensions containing ~10^8 cells $fish^{-1}$. Subsequently, 10 isolates were recognized to be harmless to the fish as there was not any sign of any pathological effects or mortalities after injection (Table 3.1). In the next experiments, these cultures were applied to diet at ~5 × 10^7 cells g^{-1} and fed to groups of fish to determine palatability. Overall, the fish accepted the modified diets and displayed a better feeding response (= stimulated appetite) than the controls. However, in a few cases the fish initially did not eat well, although with time the situation improved and the feed was consumed well.

3.1.2 Resolution of LD_{50} of the pathogen

The medium lethal doses (LD_{50}) of the two pathogens were determined to be 0.1 mL of 3 × 10^5 and 5 × 10^4 cells mL^{-1} per fish for *V. anguillarum* and *V. ordalii*, respectively. The mortalities were monitored for 14 day post-challenge.

Table 3.2 Percent mortality (%) and RPS[1] of rainbow trout fed with putative probiotics for 14 days following i.p. challenge with *V. anguillarum* or *V. ordalii*.

Bacterial culture	No. of fish	Fish challenged i.p. with:			
		V. anguillarum		*V. ordalii*	
		Mortality (%)	RPS	Mortality (%)	RPS
SL6	20	60	25	70	22
SL7	20	70	13	90	0
SL12	20	90	0	90	0
RT2	20	80	0	50	44
SM1	20	20	75	30	67
RT12	20	80	0	50	44
RT16	20	80	0	70	22
SM2	20	30	63	30	67
RT29	20	70	13	50	44
SM3	20	60	25	100	0
Control	20	80	–	90	–

[1]Relative percent survival as described by Amend (1981)

3.1.3 In vivo evaluation of putative probiotics

In preliminary small-scale experiments, groups of fish were fed with ten putative probionts in diets (~5 × 10^7 cells g^{-1}) to satiation for 14 days, and then challenged i.p. with *V. anguillarum* or *V. ordalii*. From this preliminary work, two cultures, i.e. SM1 and SM2, which were isolated from rainbow trout, were determined to be potentially beneficial against vibriosis showing a RPS value of 63–75% (Table 3.2), and were evaluated further.

3.2 Characterization and identification of bacterial isolates

3.2.1 Morphological characteristics

SM1 comprised non-motile, Gram-positive cocci which developed yellow raised colonies. In comparison, SM2 comprised non-motile, Gram-positive rods which developed orange raised colonies (Figure 3.1; Table 3.3).

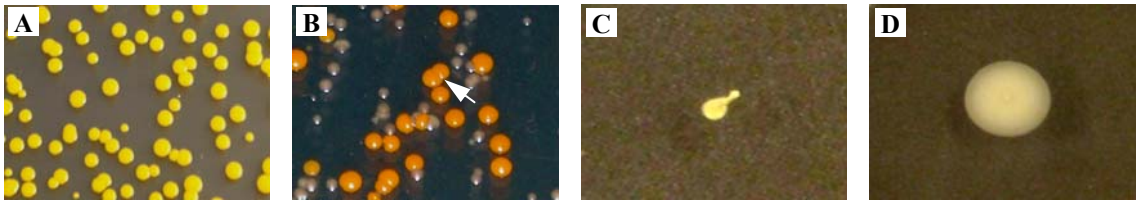

Figure 3.1 Colony morphology of probiotics – SM1 showing the presence of yellow colonies (A) and SM2 developed orange colonies (B) on TNA. Motility, which was determined by culturing bacteria on semi-solid agar plates following the measurement of the diameter of swimming zones. SM1 was non-motile (C); *V. anguillarum* was included as a positive control (D).

Table 3.3 Morphological characteristics of SM1 and SM2.

Character	SM1	SM2
Colony colour	Yellow	Orange
Cell shape	Cocci (round, smooth)	Rod (round, smooth)
Colony size	1–2 mm diameter	1–2 mm diameter
Texture	Viscous	Viscous
Motility	–	–
Gram staining reaction	+	+

3.2.2 Biochemical characteristics

Classical phenotypic tests were performed using the API 20E and 20NE system (Table 3.4), but neither of the isolates could be identified using the manufacturer's database. However, by using the diagnostic tables in Cowan and Steel (2003), SM1 and SM2 were tentatively identified as *Micrococcus* sp. and *Corynebacterium* sp., respectively.

Table 3.4 Biochemical characteristics of SM1 and SM2.

Biochemical test	SM1	SM2	Biochemical test	SM1	SM2
Oxidase	–	–	*Catalase	++	+
API 20E:			API 20NE:		
β-galactosidase	+	+	Potassium nitrate	+	–
Arginine dihydrolase	–	–	Tryptophan	–	–
Lysine decarboxylase	–	–	Glucose	–	–
Ornithine decarboxylase	–	–	Arginine	–	–
Citrate utilization	–	+	Urea	+	+
H_2S production	–	–	Aesculin	–	–
Urease production	+	+	Gelatin	+	–
Tryptophan deaminase	–	–	**pNPG	+	+
Indole production	–	–	Glucose	–	–
Voges-Proskauer reaction	–	–	Arabinose	–	+
Gelatinase production	–	–	Mannose	–	+
Glucose	–	–	Mannitol	–	+
Mannitol	–	–	N-acetyl-glucosamine	–	+
Inositol	–	–	Maltose	+	–
Sorbitol	–	–	Gluconate	–	+
Rhamnose	–	–	Caprate	–	–
Saccharose	–	–	Adipate	+	–
Melibiose	–	–	Malate	+	+
Amygdalin	–	–	Citrate	–	+
Arabinose	–	–	Phenyl-acetate	+	+

*Catalase: ++ (average); + (weak); **p-nitrophenyl-β-D-galactopyranoside

3.2.3 Physiological characteristics

SM1 and SM2 were able to grow between 4 and 37°C on TNB. Cell multiplication was also seen at 45°C for SM1 but not for SM2. Both isolates grew at pH 2–11 and in 0–20% (w/v) NaCl (Table 3.5).

Table 3.5 Physiological characteristics of the isolates.

Character	SM1	SM2
Growth temperature:		
4°C	+	+
15°C	+	+
26°C	+	+
37°C	+	+
45°C	+	–
Salinity tolerance:		
0% (w/v) NaCl	+	+
2% (w/v) NaCl	+	+
4% (w/v) NaCl	+	+
6% (w/v) NaCl	+	+
8% (w/v) NaCl	+	+
10% (w/v) NaCl	+	+
15% (w/v) NaCl	+	+
20% (w/v) NaCl	+	+
pH tolerance:		
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+

Growth was recorded as positive (+), and no growth was recorded as negative (−).

Table 3.6 Results of the API-ZYM tests for SM1 and SM2.

Enzyme assayed for:	[1]Substrate	[2]Enzyme activity of strain:	
		SM1	SM2
Control	–	–	–
Glycosidases:			
α-galactosidase	a	0	5
β-galactosidase	b	1	5
β-glucoronidase	c	0	0
α-glucosidase	d	0	2
β-glucosidase	e	0	0
N-acetyl-β-glucosaminidase	f	0	0
α-mannosidase	g	0	0
α-fucosidase	h	0	0
Peptide hydrolases:			
Leucine arylamidase	i	2	3
Valine arylamidase	j	1	1
Cystine arylamidase	k	1	0
Trypsin	l	0	1
Chymotrypsin	m	0	0
Ester hydrolases:			
Esterase (C4)	n	1	1
Esterase Lipase (C8)	o	2	2
Lipase (C14)	p	0	2
Phosphohydrolases:			
Alkaline phosphatase	q	1	0
Acid phosphatase	r	0	1
Naphthol-AS-BI-phosphohydrolase	s	1	2

[1]Substrate: [a]6-Br-2-naphthyl-αD-galactopyranoside; [b]2-naphthyl-βD-galactopyranoside; [c]Naphthol-AS-BI-βD-glucuronide; [d]2-naphthyl-αD-glucopyranoside; [e]6-Br-2-naphthyl-βD-glucopyranoside; [f]1-naphthyl-N-acetyl-βD-glucosamide; [g]6-Br-2-naphthyl-αD-mannopyranoside; [h]2-naphthyl-αL-fucopyranoside; [i]L-leucyl-2-naphthylamide; [j]L-valyl-2-naphthylamide; [k]L-cystyl-2-naphthylamide; [l]*N*-benzoyl-DL-arginine-2-naphthylamide; [m]*N*-glutaryl-phenylalanine-2-naphthylamide; [n]2-naphthyl butyrate; [o]2-naphthyl caprylate; [p]2-naphthyl myristate; [q]2-naphthyl phosphate; [r]2-naphthyl phosphate; [s]Naphthol-AS-BI-phosphate.

[2]Numbers indicate the relative magnitude of enzyme activity.

3.2.4 Enzymatic profile

Table 3.6 lists the enzymatic activities determined by use of API ZYM. SM1 was completely negative for carbohydrase activities, except for β-galactosidase production. Moreover, protease activities were weak with no activity for trypsin and chymotrypsin. Similarly, ester hydrolases and phosphohydrolases exhibited low activities, while lipase and acid phosphatase activities were not detected at all (Table 3.6). In contrast, SM2 showed more carbohydrase activities, with evidence for strong α-galactosidase and β-galactosidase production. Moderate activity was noted for α-glucosidase, but SM2 was negative for β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. There were also reasonable activities of proteases, ester hydrolases and phosphohydrolases. However, cystine arylamidase, chymotrypsin and alkaline phosphatase reactions were negative (Table 3.6).

3.2.5 Resistance to pepsin and pancreatin

As observed by growth at pH 2.0 or 3.0, the addition of pepsin at these pH values did not affect the viability of the isolates. Thus, SM1 and SM2 were resistant to pepsin at pH 2.0 and 3.0. Moreover, both isolates survived when exposed to pancreatin at pH 8.0 (Table 3.7).

Table 3.7 Effect of pepsin and pancreatin on the growth of SM1 and SM2.

Isolate	Pepsin (3 mg mL^{-1}) at:		Pancreatin (1 mg mL^{-1}) at:
	pH 2.0	pH 3.0	pH 8.0
SM1	+	+	+
SM2	+	+	+

Growth was recorded as positive (+), and no growth was recorded as negative (−).

3.2.6 Sensitivity to antibiotics

The antimicrobial sensitivity profiles of the isolates are presented in Table 3.8. SM1 was totally resistant to sulphatriad and moderately sensitive to streptomycin (= 4 mm zone of clearing). SM2 was sensitive to ampicillin, chloramphenicol and tetracycline, but resistant to penicillin and sulphatriad (Table 3.8).

Table 3.8 Antimicrobial sensitivity profiles of SM1 and SM2.

Antibiotics	Bacterial isolates	
	SM1	SM2
Ampicillin (10 µg)	S	S
Chloramphenicol (25 µg)	S	S
Penicillin G (1 IU)	S	R
Streptomycin (10 µg)	MS	MS
Sulphatriad (200 µg)	R	R
Tetracycline (25 µg)	S	S

S = sensitive; MS = moderately sensitive (≥ 4−6 mm); R = resistant

3.2.7 Production of siderophores

Bacterial siderophores are high-affinity Fe(III) chelators for the acquisition of iron under iron-limiting conditions, which could limit iron for potential pathogens. Siderophore production (= scavenging of iron) by SM1 and SM2 was determined due to the change of colour of Chrome Azurol S (CAS) agar. However, neither isolate produced siderophore (Figure 3.2).

a) b)

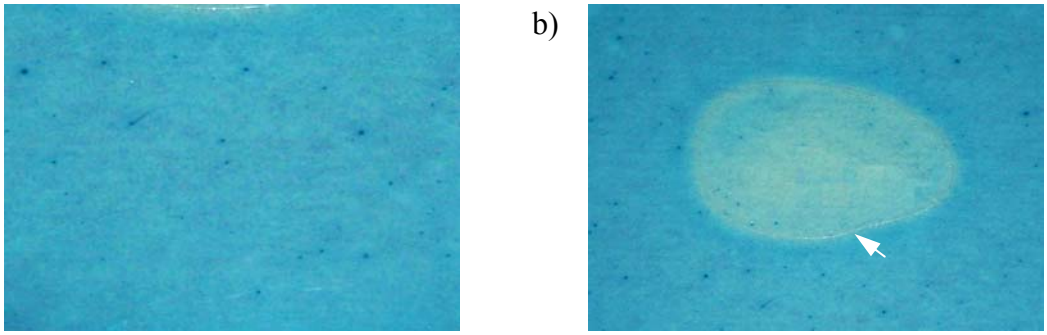

Figure 3.2 The universal CAS assay for testing siderophore production, which detects colour change of Fe-CAS complex from blue to orange (see the arrow) after chelation of the bound iron by siderophores. SM1 was negative for siderophore production (a), and *V. anguillarum* was included as positive control (b).

3.2.8 16S rDNA gene sequencing

The two isolates were identified using partial sequencing of the 16S rDNA gene (Figure 3.3) following comparison in a BLAST search, which revealed a 100% sequence homology with *Kocuria* sp. (Genbank accession number AM990819) for SM1 and

Rhodococcus sp. (Genbank accession number AY745831) for SM2, respectively. Evidently, the sequencing results for the isolates were relevant with the identification as *Micrococcus* sp. and *Corynebacterium* sp. obtained by using diagnostic tables in Cowan and Steel (2003). Indeed, the genus *Kocuria* was created from the genus *Micrococcus* (Stackebrandt *et al.* 1995). Moreover, *Corynebacterium*, which initially covered several taxa due to close relationship in 16S rRNA data sequences (see Goodfellow et al. 1998), was recently reclassified into the genera *Rhodococcus*, *Arthrobacter*, *Clavibacter*, and *Curtobacterium* (see Cowan and Steel 2003).

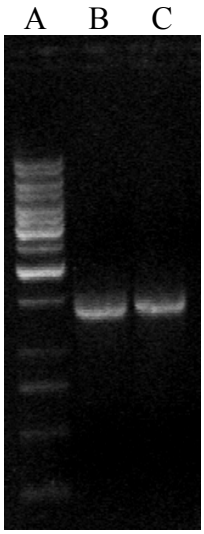

Figure 3.3 16S rDNA PCR results for the purified DNA products of probiotics – *Kocuria* SM1 (lane B) and *Rhodococcus* SM2 (lane C). Lane A: DNA ladder of 1000 base pairs.

Table 3.9 Survival of probiotic in feed maintained at different temperatures.

Probiotic	Number of viable cells on feed (cfu g^{-1})					
	Day 3		Day 7		Day 14	
	4°C	*Rt	4°C	Rt	4°C	Rt
SM1	4.7×10^7	4.2×10^7	1.2×10^7	8.8×10^6	6.7×10^6	3.5×10^6
SM2	2.6×10^7	1.5×10^7	0.2×10^7	4.5×10^6	3.6×10^6	1.2×10^6

*Room temperature

3.3 Survival of probiotics on feed

The viability of bacteria in the feed was monitored by the total viable count following storage of the diet at 4°C and room temperature (Table 3.9). When stored at room temperature, cell numbers of SM1 declined to 4.2×10^7 cfu g^{-1} from an initial 7.8×10^7 cfu g^{-1} after 3 days, and to 8.8×10^6 and 3.5×10^6 cfu g^{-1} after 7 and 14 days, respectively. However, storage at 4°C fared better with cell viability reducing to 4.7 ×

10^7, 1.2×10^7 and 6.7×10^6 cfu g^{-1} after 3, 7 and 14 days, respectively. The viability of SM2 in feed was similar to SM1 (Table 3.9). Overall, storage at 4°C prolonged the survival of cells, although a steady decrease in cell numbers for both cases occurred with both probiotics.

Table 3.10 Effect of feeding different concentrations of SM1 and SM2 on the survival of rainbow trout after i.p. challenge with *V. anguillarum* or *V. ordalii*.

SM1 in feed (cells g^{-1})	Mortality (%) after challenge with:		SM2 in feed (cells g^{-1})	Mortality (%) after challenge with:	
	[1]VIB1	[2]VIB2		[1]VIB1	[2]VIB2
~10^5	45	25	~10^5	55	45
~10^6	60	15	~10^6	35	25
~10^7	10	20	~10^7	20	25
~10^8	0	10	~10^8	40	40
~10^9	20	30	~10^9	45	65
Control	90	70	Control	75	80

[1]*V. anguillarum*; [2]*V. ordalii*

3.4 Effective feeding dose of the probiotics

The effective dose for the probiotics was determined using feed containing 10^5–10^9 cells g^{-1} feed (Table 3.10). Fish were fed with probiotic-supplement for 14 days followed by challenge with the selected pathogens, and the mortality was observed over the following 2 weeks period. The result indicated that any dose below or above 10^7 and 10^8 cells g^{-1} did not lead to good protection. SM1 conferred the best protection at a dose of ~10^8 cells g^{-1} feed, whereas the appropriate dose was ~10^7 cells g^{-1} feed for SM2.

3.5 Control of vibriosis in rainbow trout

Groups of 60 rainbow trout fed with SM1 and SM2 at doses of ~10^8 and ~10^7 cells g^{-1} of feed for 14 days followed by challenge with *V. anguillarum* or *V. ordalii* conferred marked reductions in mortalities when compared to the controls. Effectively, use of SM1 led to a significant ($P < 0.05$) reduction in mortalities, i.e. 15% (RPS = 81%) and 20% (RPS = 73%) after challenge with *V. anguillarum* and *V. ordalii*, respectively, compared to the 80 and 74% mortalities among the respective controls (Table 3.11).

Also, use of SM2 led to significant ($P < 0.05$) protection by reducing mortalities to 12% (RPS = 85) by *V. anguillarum*, and 15% (RPS = 80) after challenge with *V. ordalii* (Table 3.11). The survivors among the probiotic-fed groups were devoid of disease signs at the end of the experiments. In contrast, the surviving control fish displayed signs of disease including sluggish movement, abdominal distension, necrosis, surface haemorrhages, exophthalmia and the presence of ascitic fluid in the peritoneal cavity.

Table 3.11 Mortality (%) of rainbow trout fed with SM1 or SM2 supplemented diet for 14 days followed by i.p. challenge with *V. anguillarum* or *V. ordalii*.

Treatment	No. of fish	Rainbow trout challenged i.p. with:			
		V. anguillarum		*V. ordalii*	
		Mortality (%)	RPS	Mortality (%)	RPS
SM1	2 × 25	15 ± 10*	81	20 ± 11*	73
SM2	2 × 25	12 ± 10*	85	15 ± 5*	80
Control	2 × 25	80 ± 17	–	74 ± 15	–

Data represent the average ± SD from a triplicate set of 25 fish.

Significant difference ($P < 0.05$) from the control group is indicated by an asterisk.

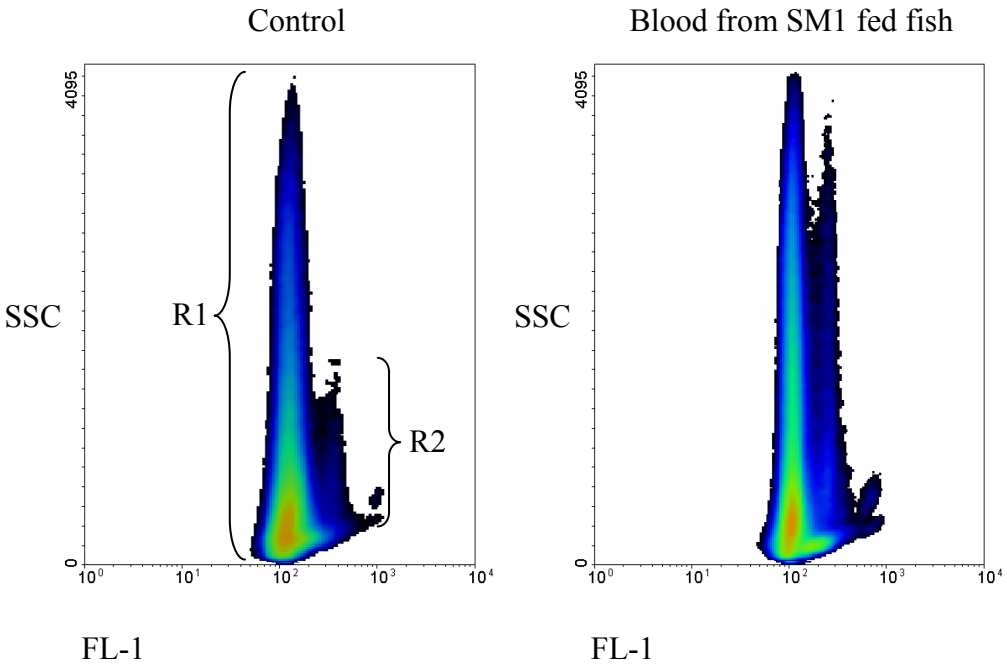

Figure 3.4 Flow cytometry analysis of DiOC6(3) stained blood cells obtained from SM1-fed and control fish. The graph illustrates the cell populations in whole blood sample (R1: erythrocytes and R2: leucocytes).

3.5.1 Mode of action

Figure 3.4 shows a typical flow cytometry analysis of whole blood cells from SM1-fed and control fish, where 'R1' was composed entirely of erythrocytes, and 'R2' was differential leucocytes according to the intensity of FL-1 and SSC properties (Newaj-Fyzul *et al.* 2007). The number of leucocytes increased significantly ($P < 0.05$) from $3.7 \pm 0.8 \times 10^6$ mL^{-1} in the controls to 5.5 ± 0.8 and $6.5 \pm 1.5 \times 10^6$ mL^{-1} in SM1 or SM2-fed fish (Table 3.12). Also, the erythrocyte counts for the probiotic treated and control fish were significantly ($P < 0.05$) elevated to 1.2 ± 0.1 and $1.1 \pm 0.1 \times 10^8$ mL^{-1} in the experimental group from $0.8 \pm 0.1 \times 10^8$ mL^{-1}, in the controls (Table 3.12). Moreover, the HK macrophage bacteriocidal activity of SM1 or SM2-fed fish was significantly higher ($P < 0.05$) than the controls with the killing index for *V. anguillarum* and *V. ordalii* calculated as 0.71 ± 0.02 or 0.73 ± 0.06, and 0.86 ± 0.05 or 0.81 ± 0.02 in treatment groups, respectively, compared with 0.87 ± 0.05 and 0.95 ± 0.04 in the corresponding controls (Table 3.12).

Table 3.12 Immunological responses of rainbow trout fed with probiotic-supplemented diet for 2 weeks.

Immunological parameter		SM1	SM2	Control
Erythrocytes ($\times 10^8$ mL^{-1})		$1.2 \pm 0.1^*$	$1.1 \pm 0.1^*$	0.8 ± 0.1
Leucocytes ($\times 10^6$ mL^{-1})		$5.5 \pm 0.8^*$	$6.5 \pm 1.5^*$	3.7 ± 0.8
Bacteriocidal activity (killing index)	[1]VIB1	$0.71 \pm 0.02^*$	$0.73 \pm 0.06^*$	0.87 ± 0.05
	[2]VIB2	$0.86 \pm 0.05^*$	$0.81 \pm 0.02^*$	0.95 ± 0.04

[1]*V. anguillarum*; [2]*V. ordalii*.

Data represent the average ± SD from a triplicate set of 10 fish.

Significant difference ($P < 0.05$) from the control group is indicated by asterisk.

Furthermore, there was a significantly ($P < 0.05$) higher respiratory burst activity of HK macrophages in the experimental (SM1: 0.05 ± 0.01; SM2: 0.04 ± 0.01) compared to the control groups (0.02 ± 0.01) (Figure 3.5). The difference between experimental and control groups was reinforced by the serum alternative complement activity, which increased from 40 ± 8 units mL^{-1} of natural haemolytic complement activity in the controls, to statistically significant ($P < 0.05$) higher levels, averaging 56 ± 7 and 61 ± 5 units mL^{-1} in the experimental group SM1 and SM2, respectively (Figure 3.6).

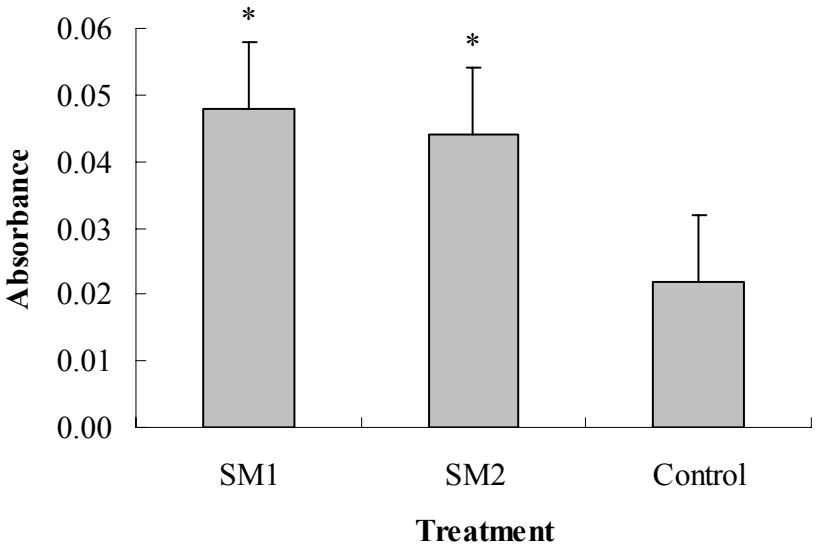

Figure 3.5 The PMA-induced respiratory burst activity of head-kidney macrophages from rainbow trout fed with probiotic-supplemented diet for 2 weeks. Data represent the average ± SD from a triplicate set of 10 fish. Significant difference ($P < 0.05$) from the control group is indicated by an asterisk.

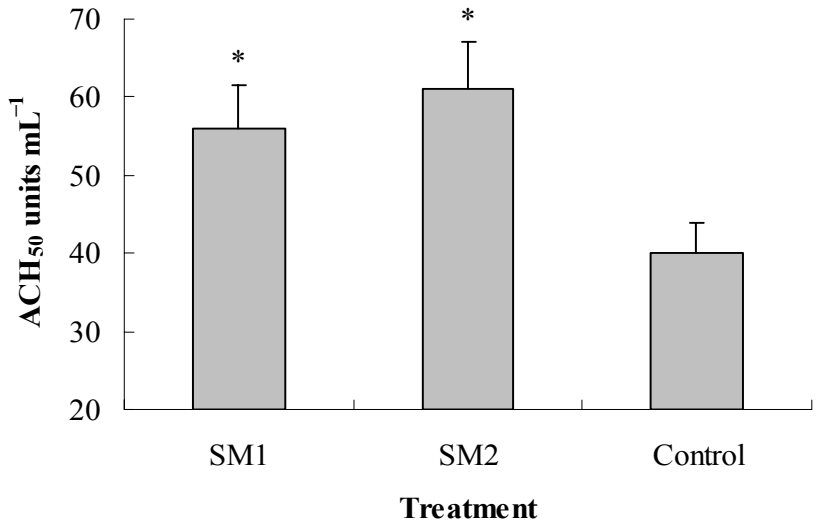

Figure 3.6 Serum alternative complement activity (ACH_{50}) in rainbow trout fed with probiotic-supplemented diets for 2 weeks. Data represent the average ± SD from a triplicate set of 10 fish. Significant difference ($P < 0.05$) from the control group is indicated by an asterisk.

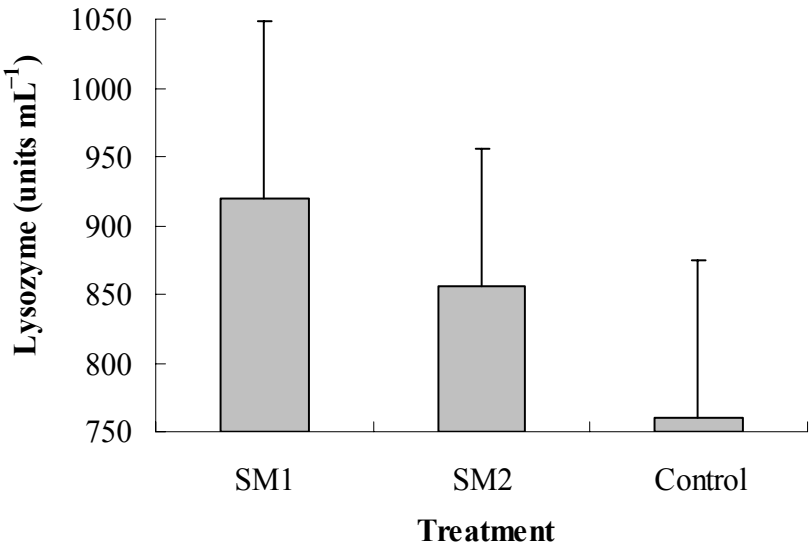

Figure 3.7 Serum lysozyme activity in rainbow trout fed with probiotic-supplemented diets for 2 weeks. Data represent the average ± SD from a triplicate set of 10 fish.

Although there was an elevation in serum lysozyme level of probiotic-fed fish, the data did not differ statistically ($P > 0.05$) to the controls. Thus, the lysozyme activity of serum was 920 ± 129, 856 ± 100 and 760 ± 115 units mL^{-1} for SM1, SM2 and control, respectively (Figure 3.7). So, the use of probiotics for 14 days led to the stimulation of cellular and humoral immune responses in rainbow trout. Certainly, immunostimulation has been identified as the dominant mode of action for a range of probiotics, including representatives of both Gram-positive and Gram-negative bacterial taxa (Austin and Austin 2007) offering host protection against pathogenic infections.

3.6 The most effective feeding duration of probiotics

When administered between one and four weeks, dietary supplementation of SM1 (~10^8 cells g^{-1} of feed) led to a reduction in mortalities after challenge with *V. anguillarum*. A two-week feeding regime with SM1 led to the maximum reduction in mortalities, i.e. 16% (RPS = 79%), compared to mortalities of 62% (RPS = 20.5%), 30% (RPS = 67%) and 22% (RPS = 69%) for one, three and four weeks feeding regimes, respectively. These compared to the 70–90% mortalities of the controls (Table 3.13). Although feeding with probiotic for one week did not result in good levels of protection, the mortalities were nevertheless significantly lower ($P < 0.05$) than the controls (Table 3.13). In general, a two-week feeding regime with SM1 led to the best protection against *V. anguillarum* in rainbow trout.

Table 3.13 Mortality (%) of rainbow trout fed with SM1 supplemented or non-supplemented control diets for up to four weeks followed by challenge with *V. anguillarum*.

Treatment	Mortality/ RPS (%)	Duration of feeding:			
		7 days	14 days	21 days	28 days
Control	Mortality	78 ± 7	77 ± 14	90 ± 10	70 ± 12
SM1	Mortality	62 ± 8^{*a}	16 ± 10^{*b}	30 ± 13^{*b}	22 ± 15^{*b}
	RPS	20.5	79	67	69

Data represent the average ± SD from a triplicate set of 20 fish.

*Different at $P < 0.05$ from the control group within the same sampling week.

Means without a common letter in a row differ significantly ($P < 0.05$) among the different probiotic feeding regimes.

3.6.1 Innate immune responses

There was a significant increase in the phagocytic activity of head kidney macrophages of fish fed SM1 at weeks two, three and four (i.e. 0.92 ± 0.06, 0.75 ± 0.03 and 0.82 ± 0.04; $P < 0.05$) compared with the levels observed in the controls, 0.60 ± 0.02 to 0.71 ± 0.04 (Figure 3.8). However, the data for week one (i.e. 0.63 ± 0.04; $P > 0.05$), which also indicated an increased activity, were not significantly different. Administration of probiotic for two weeks showed significantly higher ($P < 0.05$) phagocytic activity than all other feeding regimes (Figure 3.8). Furthermore, except for one week feeding with SM1 (0.68 ± 0.02; $P > 0.05$), the peroxidase content of the macrophages from experimental groups, i.e. 0.91 ± 0.04, 0.85 ± 0.04 and 0.89 ± 0.01 for two, three and four weeks, respectively, was statistically different ($P < 0.05$) from that of control fish, i.e. averaging 0.66 ± 0.02 (Figure 3.9). Moreover, there was significantly elevated ($P < 0.05$) serum total antiprotease activity after two weeks of probiotic treatment (Figure 3.10). In contrast, the mean antiproteases for other feeding periods, which ranged from 72.4 ± 4.0 to 80.7 ± 2.5, did not differ statistically but still sustained high levels as compared to the controls (68.0 ± 2.0 to 74.4 ± 3.5) (Figure 3.10). Moreover, the lysozyme activity of serum was between 850 and 1350 units mL^{-1} for controls, and 900–1850 units mL^{-1} for the fish fed with probiotic. In particular, two weeks of probiotic feeding demonstrated significantly increased ($P < 0.05$) activity in comparison with the controls (Figure 3.11).

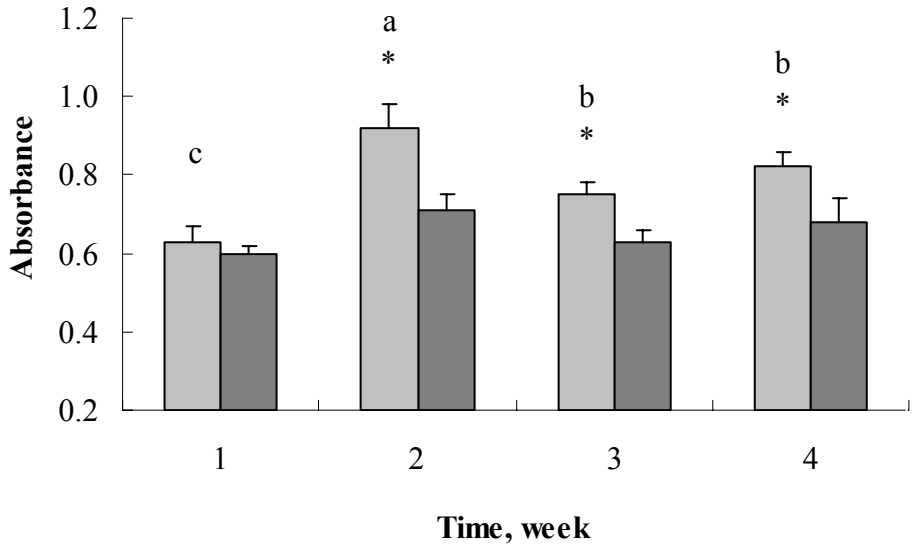

Figure 3.8 Phagocytic activity ($OD_{510\ nm}$) of rainbow trout head kidney macrophage from specimens fed SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a duplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) among the different probiotic feeding regimes.

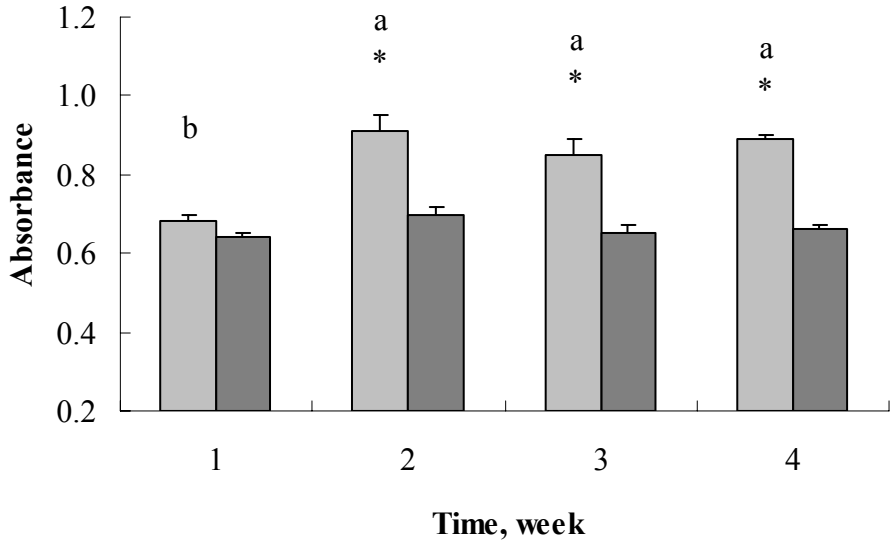

Figure 3.9 Head kidney macrophage peroxidase content ($OD_{450\ nm}$) in rainbow trout fed SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a duplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) among the different probiotic feeding regimes.

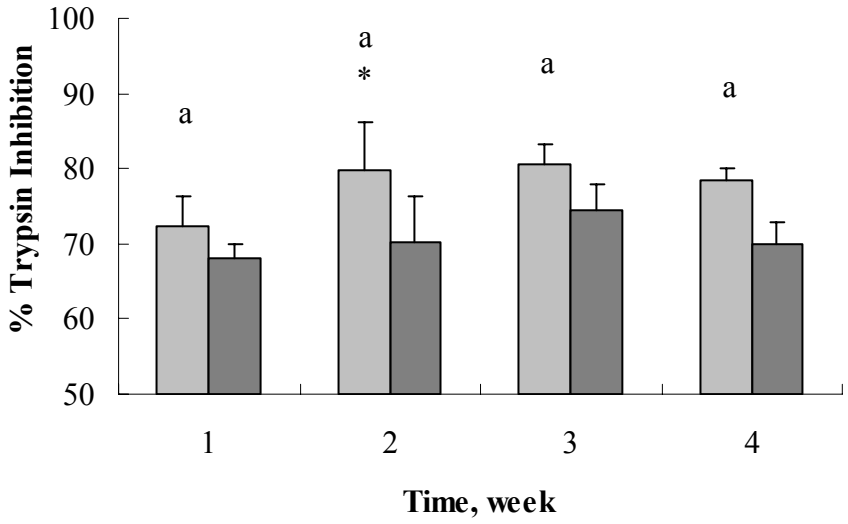

Figure 3.10 Serum total antiproteases activity in rainbow trout fed SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a triplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) among the different probiotic feeding regimes.

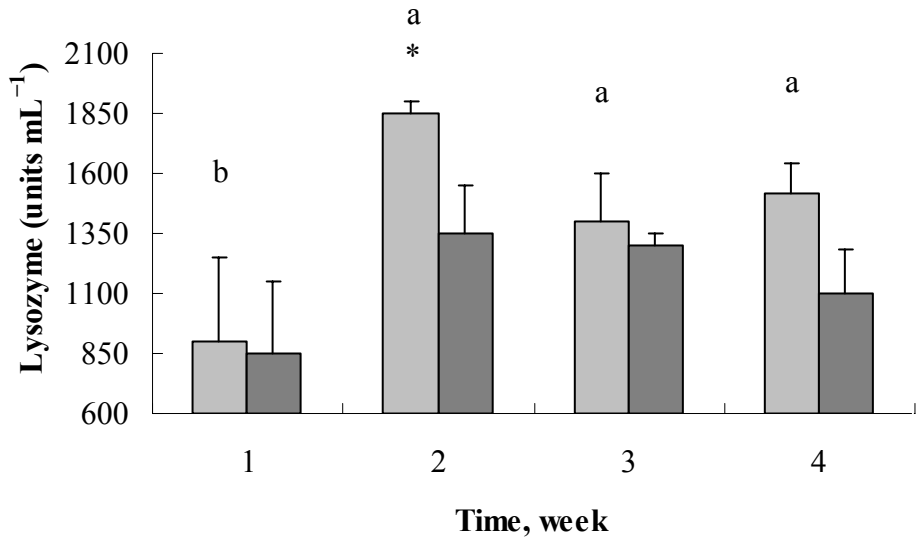

Figure 3.11 Serum lysozyme activity in rainbow trout fed SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a triplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) among the different probiotic feeding regimes.

Conversely, the data did not reveal any significant differences for blood respiratory burst activity between experimental (0.20 ± 0.02 to 0.23 ± 0.05) and controls

(0.16 ± 0.01 to 0.19 ± 0.04), but the levels of respiratory burst following probiotic treatments remained enhanced over the controls until the end of the experiment (Figure 3.12). So, a two-week feeding regime with SM1 was most immunomodulatory leading to significant stimulation of macrophage phagocytic activity compared to other treatments and controls. Moreover, in comparison with the controls, serum antiprotease and lysozyme activities were significantly enhanced at week two. Likewise, there was a pronounced effect on macrophage peroxidase at the end of week two.

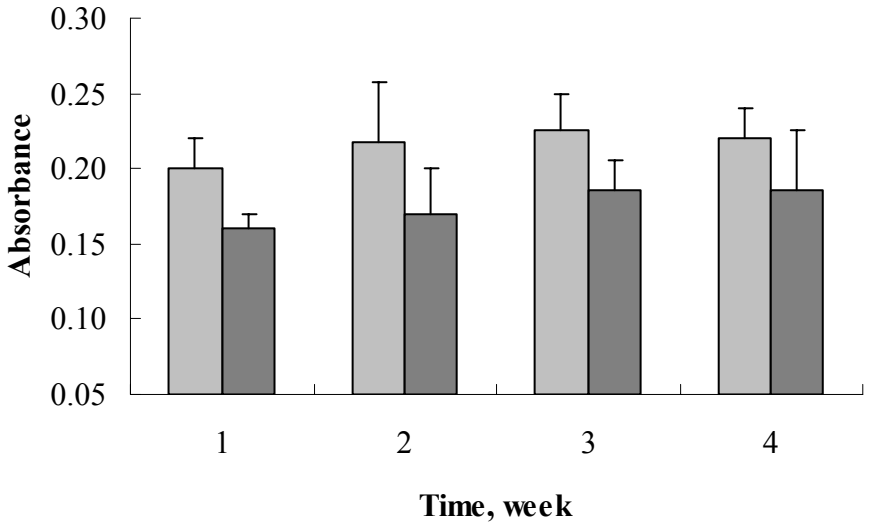

Figure 3.12 Blood respiratory burst activity ($OD_{550\ nm}$) in rainbow trout fed SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a triplicate set of 10 fish.

3.7 Synergistic effects of probiotics

An equi-mixture of SM1 (5×10^7 cells g^{-1}) and SM2 (5×10^7 cells g^{-1}) conferred significant ($P < 0.05$) protection against vibrio infections when fed to rainbow trout for 14 days as feed supplement, i.e. a reduction in mortalities to 20–27% (RPS = 68–74%) after challenge with *V. anguillarum* or *V. ordalii* compared to 78–84% mortalities of the controls (Table 3.14). Although, the use of a mixture of probiotic bacteria significantly enhanced fish survival compared to the controls, the beneficial effect was not statistically different (data not shown) when compared to the application of single culture of probiotic (see Section 3.5).

Table 3.14 Mortality (%) of rainbow trout fed an equi-mixture of SM1 and SM2 supplemented diet for 14 days following by challenge with *V. anguillarum* or *V. ordalii*.

Treatment	Rainbow trout challenged i.p. with:			
	V. anguillarum		*V. ordalii*	
	Mortality (%)	RPS	Mortality (%)	RPS
SM1+SM2	20 ± 16*	74	27 ± 6*	68
Control	78 ± 13	–	84 ± 14	–

Data represent the average ± SD from a triplicate set of 25 fish.

Significant difference ($P < 0.05$) from the control group is indicated by an asterisk.

3.7.1 Changes in immune parameters

The data for intracellular respiratory burst activity of HK macrophages, which was detected from the reduction of NBT to formazan as a measure of superoxide anion ($O2^{-}$) production, was statistically significant ($P < 0.05$) with groups fed with mixture of probiotics supplemented diets (0.06 ± 0.01) relative to the controls (0.02 ± 0.01) (Table 3.15). Moreover, the use of mixed probiotics led to significantly pronounced macrophage phagocytosis, i.e. 0.75 ± 0.07 ($P < 0.05$), compared with the controls, 0.44 ± 0.03 (Table 3.15). Although the serum peroxidase activity and total immunoglobulin levels were higher than the controls, the data were not significantly different ($P > 0.05$). The peroxidase activities were 0.38 ± 0.03 and 0.33 ± 0.05, and immunoglobulin levels were 28 ± 3.5 mg mL^{-1} and 26 ± 2.5 mg mL^{-1} for experimental and control fish, respectively (Table 3.15).

Table 3.15 Immunological responses of rainbow trout fed an equi-mixture of SM1 and SM2 supplemented diet for 2 weeks.

Immunological parameter	SM1 + SM2	Control
O^{2-} production ($OD_{620\ nm}$)	0.06 ± 0.01*	0.02 ± 0.01
Phagocytosis ($OD_{510\ nm}$)	0.75 ± 0.07*	0.44 ± 0.03
Peroxidase ($OD_{550\ nm}$)	0.38 ± 0.03	0.33 ± 0.05
Total immunoglobulin (mg mL^{-1})	28 ± 3.5	26 ± 2.5

Data represent the average ± SD from a triplicate set of 10 fish.

Significant difference ($P < 0.05$) from the control group is indicated by asterisk.

3.8 Long-term beneficial effects of probiotics

Following a 14 day feeding regime with probiotics, rainbow trout were challenged at weekly intervals for 5-weeks with the data revealing a significantly ($P < 0.05$) lower mortality for up to four weeks compared with the untreated controls. Thus, the mortality rate of fish fed SM1 was reduced to 10 ± 3% ($P < 0.05$) at one week after probiotic feeding compared to mortalities of 27 ± 8 ($P < 0.05$), 24 ± 15 ($P < 0.05$) and 28 ± 17% ($P < 0.05$) after two, three and four weeks, respectively. These compared to 73 ± 6 to 92 ± 3% mortalities of the controls (Table 3.16). In contrast, there was no significant difference ($P > 0.05$) in the mortality among fish groups (SM1: 50 ± 15%; control: 78 ± 25%) after the challenge at five weeks. Use of SM1 resulted in a RPS of 87, 71, 68, 62 and 36% against *V. anguillarum* infection after one, two, three, four and five weeks, respectively, representing a sign of gradual decrease in survival (Table 3.16).

Table 3.16 Mortality (%) and RPS following i.p. challenge with *V. anguillarum* in groups fed with SM1 or control diets.

Treatment	Mortality / RPS (%)	Mortalities (%) after challenge 7-35 days after feeding with probiotic for 14-days:				
		7-days	14-days	21-days	28-days	35-days
Control	Mortality	79 ± 9	92 ± 3	76 ± 5	73 ± 6	78 ± 25
SM1	Mortality	10 ± 3^{a*}	27 ± 8^{ab*}	24 ± 15^{ab*}	28 ± 17^{ab*}	50 ± 15^{b}
	RPS	87	71	68	62	36

Data represent the average ± SD from a triplicate set of 20 fish.

Significant difference ($P < 0.05$) from the control group is indicated by an asterisk.

Means without a common superscript lower-case letter in a row differ significantly ($P < 0.05$) among the probiotic treated groups.

3.8.1 Protective immunity

At the end of two weeks after feeding with probiotic SM1, the fish showed a significantly enhanced respiratory burst activity of blood (0.07 ± 0.01; $P < 0.05$), although the levels after three (0.05 ± 0.01), four (0.05 ± 0.01) and five weeks (0.04 ± 0.01) did not differ statistically ($P > 0.05$) compared to the controls (0.04 ± 0.00 to 0.05 ± 0.01) (Table 3.17). Moreover, compared to controls (605 ± 185 units mL^{-1}), probiotic-

fed fish exhibited significantly improved serum lysozyme activity (872 ± 114 units mL^{-1}; $P < 0.05$) only at two weeks post-feeding. Thereafter, serum lysozyme activity, although higher, was not different ($P > 0.05$) than that of the control (Table 3.17). Particularly, there was significantly less activity at week five (497 ± 100 units mL^{-1}; $P < 0.05$) when compared within the probiotic treated samples (Table 3.17). Furthermore, the leukocrit content, i.e. white blood cell numbers (% WBC) remained statistically non-significant ($P > 0.05$) among fish groups throughout the experimental period, except with fish fed SM1 (5.1 ± 1.1%; $P < 0.05$) after three weeks (Table 3.17). The corresponding serum peroxidase and bacterial killing activities three weeks after ending the probiotic feeding regime were significantly higher [i.e. (0.37 ± 0.11; $P < 0.05$) and (percentage of surviving bacteria = 57 ± 4%; $P < 0.05$), respectively], compared with 0.23 ± 0.02 and 82 ± 12% of the controls. However, these immune parameters for the other sampling periods remained statistically similar ($P > 0.05$), although higher values were noted for the SM1 group (Figures 3.13, 3.14). These observations indicate that, there was a steady maintenance of immune response from the induction phase, i.e. there may be an innate memory responsible for long-term protection, which was decreased over time.

Table 3.17 Changes in immune parameters of rainbow trout over five weeks after feeding with probiotic SM1.

Character	Treatment	Weeks after cessation of probiotic			
		W-2	W-3	W-4	W-5
[1]R. burst	SM1	0.07 ± 0.01[a*]	0.05 ± 0.01[b]	0.05 ± 0.01[b]	0.04 ± 0.01[b]
	Control	0.05 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
[2]Lysozyme	SM1	872 ± 114[a*]	755 ± 168[ab]	695 ± 156[b]	497 ± 100[c]
	Control	605 ± 185	718 ± 133	633 ± 112	450 ± 93
[3]Leukocrit	SM1	4.2 ± 1.4[a]	5.1 ± 1.1[a*]	4.5 ± 2.0[a]	3.3 ± 1.3[a]
	Control	2.3 ± 0.5	2.9 ± 1.8	3.6 ± 0.6	3.1 ± 0.8

[1]Respiratory burst ($OD_{550\ nm}$); [2]Serum lysozyme (units mL^{-1}); [3]Leukocrit (% WBC)

Data represent the average ± SD from a triplicate set of 10 fish.

[*]Data in the same column are significantly different ($P < 0.05$) from the control.

Significant differences ($P < 0.05$) following the probiotic treatment are indicated with different superscript lower-case letter.

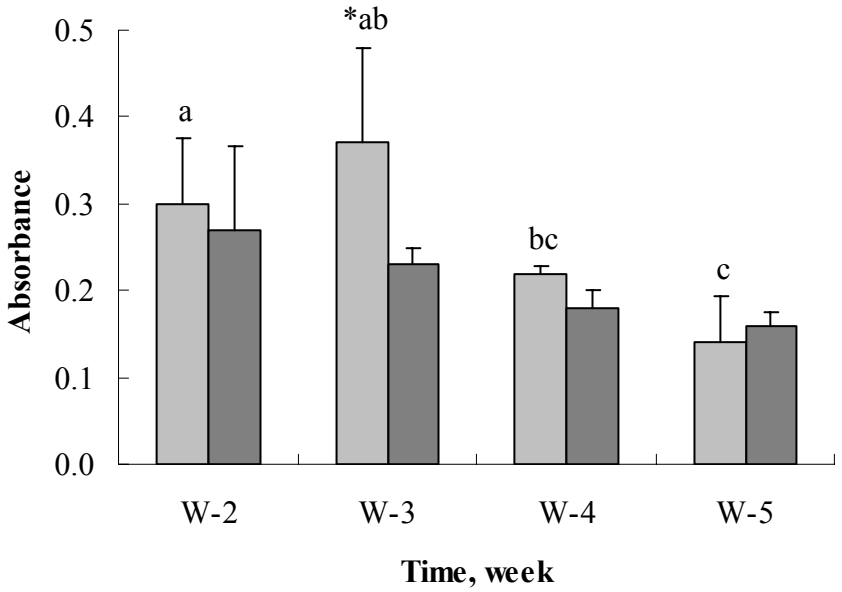

Figure 3.13 Serum peroxidase activity of rainbow trout over five weeks after feeding with probiotic SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a triplicate set of 10 fish. *Different at $P < 0.05$ level from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) following the probiotic treatment at different time points.

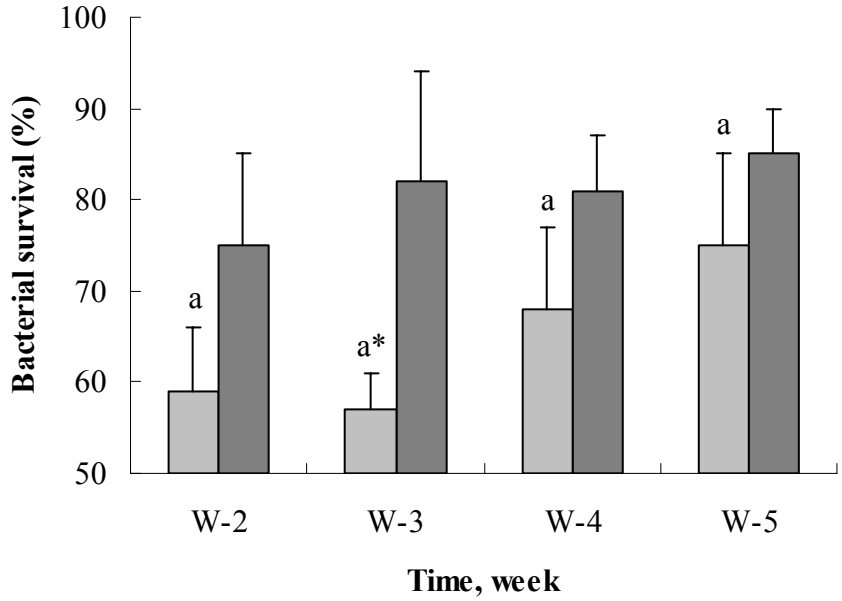

Figure 3.14 Survival percentage (%) of *V. anguillarum* incubated with rainbow trout serum collected over five weeks after feeding with *Kocuria* SM1 supplemented (□) or non-supplemented control (■) diets. Data represent the average ± SD from a triplicate set of 10 fish. *Different at $P < 0.05$ level from the control group within the same sampling week. Means without a common letter differ significantly ($P < 0.05$) following the probiotic treatment at different time points.

3.9 Protective efficacy of cell components of probiotics

The viable cells of probiotics SM1 and SM2 were highly effective in controlling vibriosis in rainbow trout. Here, I investigated the efficacy of sub-cellular components of bacterial cultures to determine which part of the cell is responsible for protection against vibriosis in rainbow trout. Thus, fish inoculated with extracellular proteins (ECPs), cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2, respectively, followed by challenge on day 8 with *V. anguillarum* led to 11–38% mortalities compared with 86% mortalities in the controls (Figure 3.15). In particular, use of CWPs (SM1: 17%; SM2: 14%) and WCPs (SM1: 13%; SM2: 11%) of the probiotics led to significant ($P < 0.05$) resistance to infection. However, the total mortalities for the ECPs did not differ statistically when compared to controls (Figure 3.15). Overall, these results pointed to the potential of using cellular components of probiotics in controlling bacterial fish diseases and may well explain the parts of the cells involved in protection. Moreover, SDS-PAGE profiles of WCPs of probiotics revealed 23–26 protein bands (range: 17.3 to 209 kDa) in comparison to 11–12 well stained bands between 13.1 to 209 kDa in the CWPs. This compares with 6–8 bands (range: 22 to 182 kDa) for the ECPs (Figures 3.16, 3.17). Some likely common proteins (see arrows on gel image) were also clear between WCPs and CWPs for both probiotics.

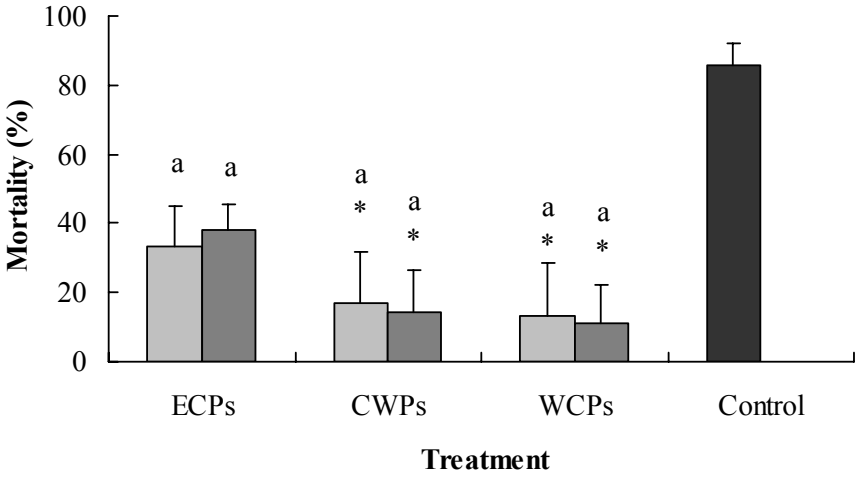

Figure 3.15 Percent mortality (%) of rainbow trout following i.p. challenge with *V. anguillarum* after inoculation with cellular components of the probiotics SM1 (□) or SM2 (■) for 7 days, compared with controls (■). Data represent the average ± SD from a triplicate set of 10 fish. *Significantly different ($P < 0.05$) from the control group. Means with a common letter did not differ significantly ($P > 0.05$) among different treatments with cellular proteins of the probiotics.

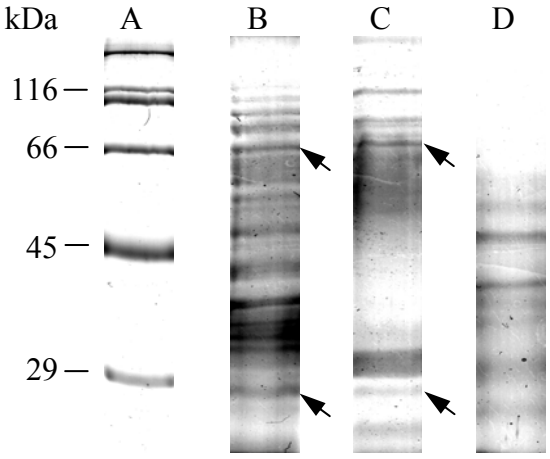

Figure 3.16 Coomassie brilliant blue stained SDS-PAGE analysis of proteins extracted from the probiotic SM1. Lanes: (A) protein markers, (B) whole cell proteins (WCPs), (C) cell wall proteins (CWPs) and (D) extra cellular proteins (ECPs). Arrows points to likely common proteins.

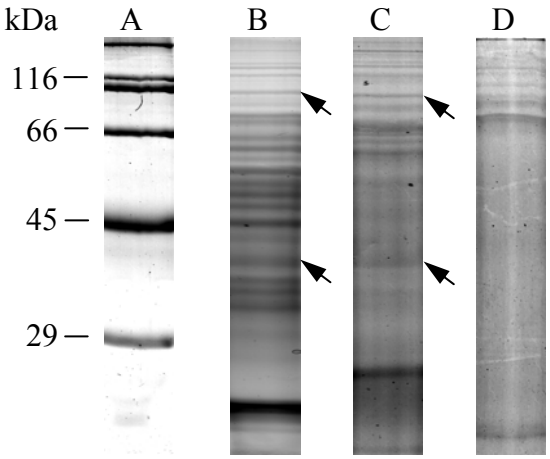

Figure 3.17 Coomassie brilliant blue stained SDS-PAGE analysis of proteins extracted from the probiotic SM2. Lanes: (A) protein markers, (B) whole cell proteins (WCPs), (C) cell wall proteins (CWPs) and (D) extra cellular proteins (ECPs). Arrows points to likely common proteins.

3.9.1 Mode of action

A significant ($P < 0.05$) influence in the respiratory burst activity, i.e. production of superoxide anion was observed in groups receiving CWPs and WCPs, with the highest level 0.045 ± 0.002 in fish inoculated with WCPs of SM2 when compared to the controls, 0.039 ± 0.003. Although the difference was non-significant ($P > 0.05$), the

level of induction was maintained at elevated levels with ECPs (Figure 3.18). The serum peroxidase activity was significantly different ($P < 0.05$) from the controls in experimental samples except those inoculated with ECPs (Figure 3.19).

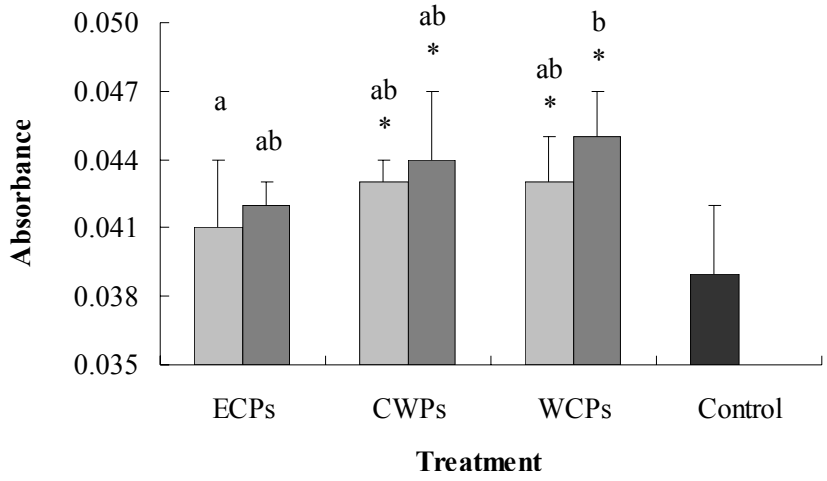

Figure 3.18 Blood respiratory burst activity in rainbow trout inoculated with cellular components of the probiotics SM1 (□) or SM2 (■), compared with controls (■). *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ at $P < 0.05$ among different treatments with cellular proteins of probiotics.

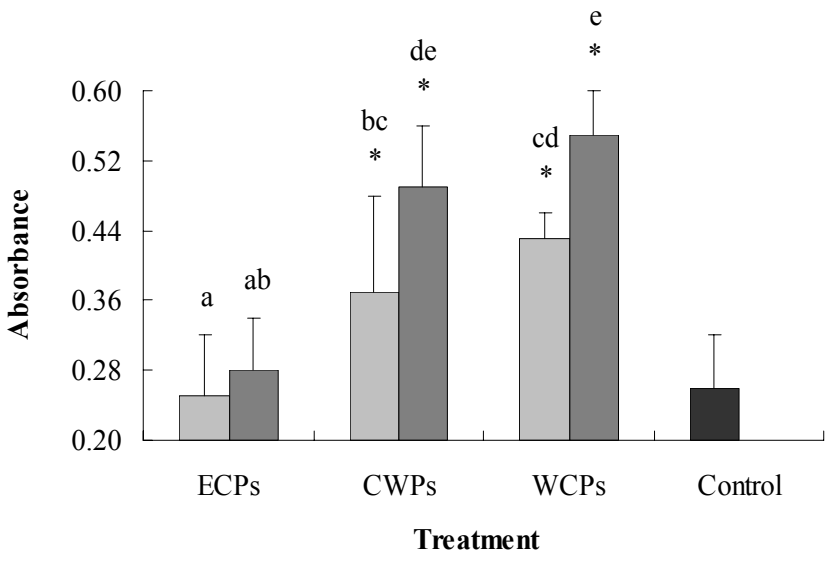

Figure 3.19 Serum peroxidase activity of rainbow trout inoculated with cellular components of the probiotics SM1 (□) or SM2 (■), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

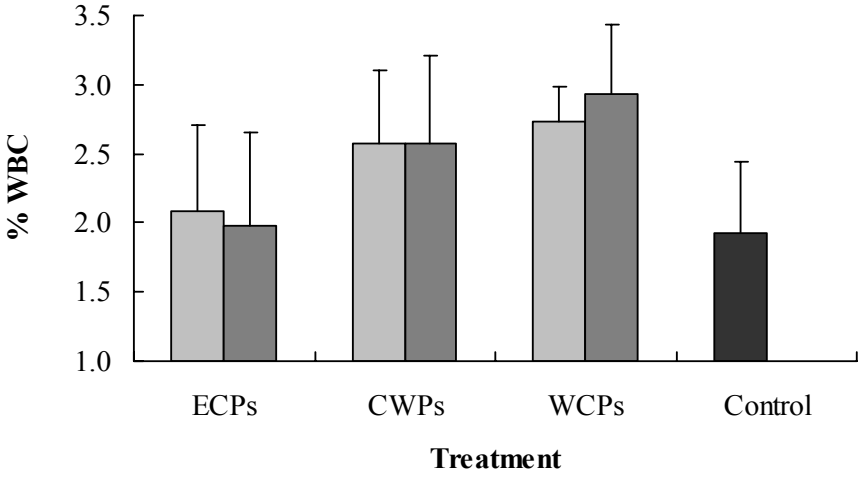

Figure 3.20 The leukocrit content, i.e. white blood cell numbers (% WBC) in rainbow trout inoculated with cellular components of the probiotics SM1 (□) or SM2 (■), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

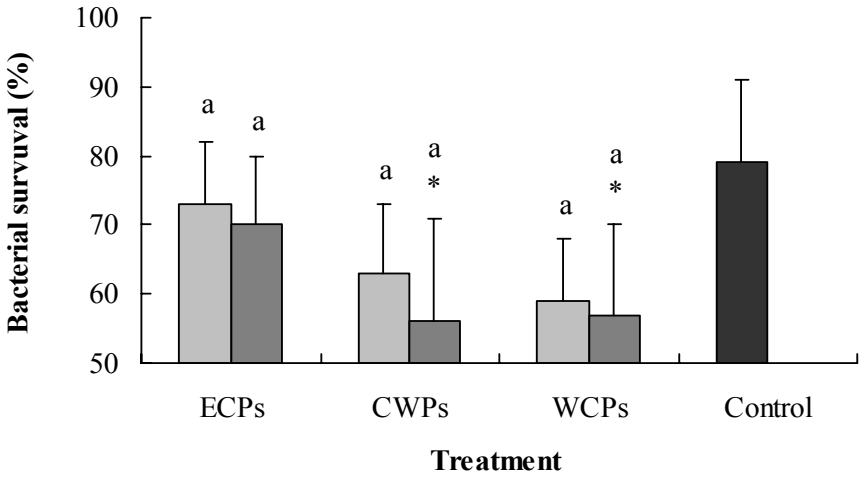

Figure 3.21 Survival percentage (%) of *V. anguillarum* incubated with serum of rainbow trout inoculated with cellular components of the probiotics SM1 (□) or SM2 (■), compared with controls (■). Data represent the average ± SD from a triplicate set of 5 fish. *Significantly different ($P < 0.05$) from the control group. Means without a common letter differ significantly ($P < 0.05$) among different treatments with cellular proteins of probiotics.

Moreover, all groups of fish inoculated with cellular proteins of probiotics had an increased ($P > 0.05$) number of white blood cells between 1.98 ± 0.67% and 2.93 ±

0.51%, as examined by measurement of the leukocrit value, compared with the controls (1.93 ± 0.51%) (Figure 3.20). However, significantly ($P < 0.05$) enhanced bacteriocidal activity was recorded following inoculation with CWPs and WCPs of SM2 compared with the controls. Improved bacterial killing was also noted in the rest of the treated groups compared to the controls, but the differences were not significant (Figure 3.21).

Table 3.18 Growth performance, feed utilization and body indices of rainbow trout fed with probiotic-supplemented or control diets.

Character	14 days feeding regime with:		
	SM1	SM2	Control
[1]Weight gain (%)	20.3 ± 8.7[a]	25.2 ± 6.2[a]	23.3 ± 4.5[a]
[2]SGR (% day^{-1})	1.4 ± 0.8[a]	1.9 ± 0.6[a]	1.6 ± 0.2[a]
[3]FCR	0.29 ± 0.01[a]	0.27 ± 0.02[a]	0.29 ± 0.01[a]
[4]PER	1.5 ± 0.5[a]	2.3 ± 0.7[a]	1.6 ± 0.3[a]
[5]HSI (%)	1.3 ± 0.1[a]	1.5 ± 0.0[a]	1.4 ± 0.2[a]
[6]SMI (%)	0.19 ± 0.05[a]	0.17 ± 0.06[a]	0.18 ± 0.02[a]

[1][(Final weight – Initial weight) ÷ Initial weight] × 100.

[2][(ln Final weight – ln Initial weight) ÷ Experiment duration (days)] × 100.

[3][Total feed consumed (g) ÷ Total wet weight gain of animal (g)].

[4][Total wet weight gain of animal (g) ÷ Total protein intake (g)].

[5][Weight of liver (g) ÷ Weight of whole fish (g)] × 100.

[6][Weight of spleen (g) ÷ Weight of whole fish (g)] × 100.

Data represent the average ± SD from a triplicate set of 25 fish.

Means without a common superscript lower-case letter in a row differ significantly ($P < 0.05$) among the treatments.

3.10 Growth performance of fish

Results of the growth performance, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and body indices [hepato-somatic index (HSI) and spleen mass index (SMI)] measured per treatment are summarized in Table 3.18. Fish biomass increased by ~20–25% in all groups after the 14 day feeding regime, with SGR

≤ 1.90% and FCR ≥ 0.27. Compared to the control group, SGR was improved from 1.6 ± 0.2 to 1.9 ± 0.6%, and FCR was reduced from 0.29 ± 0.01 to 0.27 ± 0.02 in the group SM2. The PER was also enhanced in SM2 supplemented group (2.3 ± 0.7) compared to the controls (1.6 ± 0.3). However, no significant differences ($P > 0.05$) among the treatments were observed for any of the parameters, including the body indices.

3.11 Influence on digestive enzymes

Semiquantitative API ZYM assays resulted in no differences in the activity of ester hydrolases or phosphohydrolases of rainbow trout fed with probiotics or control diets (Table 3.19). Moreover, the activity of proteases was mostly similar between groups, except for relatively weak reaction of trypsin and leucine arylamidase with the SM1 and controls, respectively (Table 3.19). On the other hand, variable results were observed for carbohydrases activities. Notably, α-mannosidase activity was strong with fish fed SM1 followed by moderate and fair activity with SM2 and control fish. Higher α-fucosidase and α-glucosidase activity was recorded for SM1 and control groups. In contrast, β-glucoronidase activity was lower after feeding with SM2 (Table 3.19).

3.12 Changes in biochemical indicators

As part of a general health assessment, modifications of serum biochemistry parameters, i.e. total protein, albumin, globulin, haemoglobin (Hb), glucose, urea, and creatinine were measured after fish were fed for 14 days with probiotic-supplemented or control diets. The total serum protein level differed significantly ($P < 0.05$) between experimental group (SM1: 23.0 ± 4.4 mg mL^{-1}; SM2: 25.0 ± 1.0 mg mL^{-1}) and the controls (16.0 ± 1.3 mg mL^{-1}) (Table 3.20). Similarly, there was significantly ($P < 0.05$) increased albumin (7.3 ± 0.2 and 8.2 ± 0.2 mg mL^{-1} of the SM1 and SM2 treatments compared to 6.1 ± 0.1 mg mL^{-1} of the controls) and globulin content (15.7 ± 0.2 and 16.8 ± 0.2 mg mL^{-1} of the SM1 and SM2 treatments compared to 9.9 ± 0.1 mg mL^{-1} of the controls) in the serum of rainbow trout fed the probiotic SM1 or SM2 diets compared to the controls (Table 3.20). However, the levels of haemoglobin, and urea and creatinine (= marker of kidney function) were not affected by any of the experimental diets (Table 3.20). Serum glucose, which is often used as an indicator of stress, also remained statistically similar ($P > 0.05$) between groups (Table 3.20).

Table 3.19 Results of API ZYM test 2 h after last meal, carried out on homogenates of digestive organs with digesta of juvenile rainbow trout.

Enzyme assayed for:	[1]Substrate	[2]Enzyme activity of treatment:		
		SM1	SM2	Control
Control	–	–	–	–
Glycosidases:				
α-galactosidase	a	0	0	0
β-galactosidase	b	5	5	5
β-glucoronidase	c	4	2	4
α-glucosidase	d	2	2	3
β-glucosidase	e	0	0	0
N-acetyl-β-glucosaminidase	f	5	5	5
α-mannosidase	g	5	3	2
α-fucosidase	h	3	2	2
Peptide hydrolases:				
Leucine arylamidase	i	5	5	4
Valine arylamidase	j	5	5	5
Cystine arylamidase	k	1	1	1
Trypsin	l	3	5	5
Chymotrypsin	m	0	0	0
Ester hydrolases:				
Esterase (C4)	n	2	2	2
Esterase Lipase (C8)	o	2	2	2
Lipase (C14)	p	0	0	0
Phosphohydrolases:				
Alkaline phosphatase	q	5	5	5
Acid phosphatase	r	5	5	5
Naphthol-AS-BI-phosphohydrolase	s	5	5	5

[1]Substrate: [a]6-Br-2-naphthyl-αD-galactopyranoside; [b]2-naphthyl-βD-galactopyranoside; [c]Naphthol-AS-BI-βD-glucuronide; [d]2-naphthyl-αD-glucopyranoside; [e]6-Br-2-naphthyl-βD-glucopyranoside; [f]1-naphthyl-N-acetyl-βD-glucosamide; [g]6-Br-2-naphthyl-αD-mannopyranoside; [h]2-naphthyl-αL-fucopyranoside; [i]L-leucyl-2-naphthylamide; [j]L-valyl-2-naphthylamide; [k]L-cystyl-2-naphthylamide; [l]*N*-benzoyl-DL-arginine-2-naphthylamide; [m]*N*-glutaryl-phenylalanine-2-naphthylamide; [n]2-naphthyl butyrate; [o]2-naphthyl caprylate; [p]2-naphthyl myristate; [q]2-naphthyl phosphate; [r]2-naphthyl phosphate; [s]Naphthol-AS-BI-phosphate.

Homogenate concentration was adjusted to 1 mg mL^{-1} protein in all cases.

[2]Numbers indicate the relative magnitude of enzyme activity.

Table 3.20 Serum biochemical indicators of rainbow trout fed with probiotic-supplemented or control diets for 14 days.

Character	SM1	SM2	Control
Protein (mg mL^{-1})	23.0 ± 4.4*	25.0 ± 1.0*	16.0 ± 1.3
Albumin (mg mL^{-1})	7.3 ± 0.2*	8.2 ± 0.2*	6.1 ± 0.1
Globulin (mg mL^{-1})	15.7 ± 0.2*	16.8 ± 0.2*	9.9 ± 0.1
Haemoglobin (mg mL^{-1})	5.0 ± 0.9	5.4 ± 1.5	4.5 ± 1.6
Urea (mg mL^{-1})	0.08 ± 0.01	0.10 ± 0.02	0.10 ± 0.01
Creatinine (mg mL^{-1})	0.015 ± 0.005	0.019 ± 0.006	0.018 ± 0.006
Glucose (mg mL^{-1})	1.09 ± 0.03	1.07 ± 0.05	1.01 ± 0.08

Data represent the average ± SD from a triplicate set of 10 fish.

Significant difference ($P < 0.05$) from the control group is indicated by asterisk.

CHAPTER 4 – DISCUSSION

4.1 Discussion

Outbreaks of infectious bacterial diseases remain a key limitation to the expansion of fish and shellfish aquaculture, worldwide (Subasinghe 2005; Austin and Austin 2007). Recently, farmed Atlantic salmon, Pacific salmon and rainbow trout in the South of Chile have suffered from high mortalities due to vibriosis (Colquhoun *et al.* 2004; Silva-Rubio *et al.* 2008a,b). Vibriosis, which is attributable to *V. anguillarum* and *V. ordalii*, is an acute haemorrhagic septicaemia for which current control strategies centre on vaccination and chemotherapy (Austin and Austin 2007). However, the use of antibiotics may lead to the development and spread of antibiotic-resistant bacteria, which may pose a risk to human health (Alcaide *et al.* 2005; Akinbowale *et al.* 2006). Thus, the value of preventative measures is recognized. As a consequence, the use of probiotics, which were initiated during the late 1980s (Dopazo *et al.* 1988; Kamei *et al.* 1988), has garnered attention (Figure 4.1) for disease prevention in aquaculture (Austin *et al.* 1995; Gildberg *et al.* 1997; Gatesoupe 1999; Verschuere *et al.* 2000; Spanggaard *et al.* 2001; Irianto and Austin 2002a,b; Balcázar *et al.* 2006a; Gobeli *et al.* 2009).

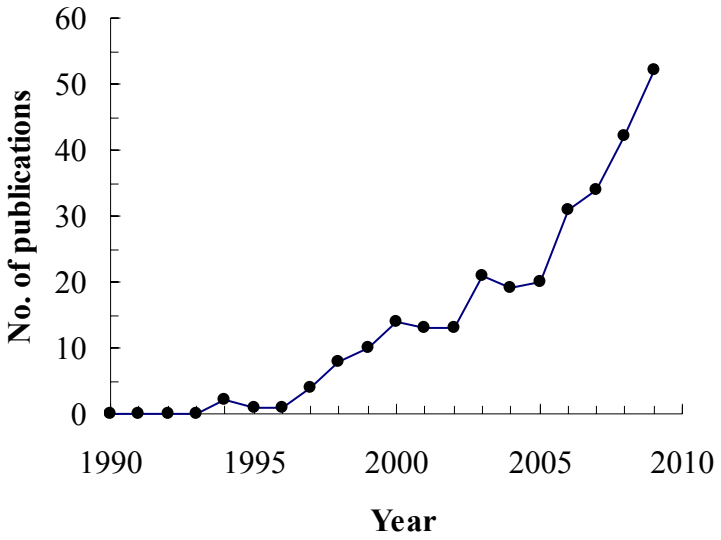

Figure 4.1 Interest in probiotics for use in aquaculture. Source: search on ISI Web of Knowledge (www.isiknowledge.com) using term "probiotic + aquaculture".

To date, the use of probiotics has led to control of a range of bacterial pathogens in various fish species. For example, supplementation of *La. lactis* subsp. *lactis*, *Leu. mesenteroides* and *L. sakei* dosed at 10^6 cfu g^{-1} feed for two weeks significantly improved the survival rate by 32.2–34.2% of rainbow trout after challenge with *A. salmonicida* (Balcázar *et al.* 2007b). Moreover, rainbow trout were protected against *A.*

salmonicida and *Y. ruckeri* when administered with dietary *C. maltaromaticum* and *C. divergens* at $>10^7$ cells g^{-1} for two weeks (Kim and Austin 2006a). Similarly, reduction of *La. garvieae*, *V. anguillarum*, *A. hydrophila*, *A. sobria* and *Streptococcus* sp. associated mortalities in rainbow trout, Chinese drum (*M. miiuy*), Nile tilapia (*O. niloticus*), perch (*P. fluviatilis*) and grouper (*E. coioides*) was noticeable by the use of probiotics, i.e. *A. sobria* (Brunt and Austin 2005), *C. butyricum* (Pan *et al.* 2008), *M. luteus* (El-Rhman *et al*. 2009), *Ps. chlororaphis* (Gobeli *et al.* 2009) and *L. plantarum* (Son *et al.* 2009), respectively. Overall, probiotics have been attributed with improved nutrition (Balcázar *et al.* 2006a) and food safety in a more environmentally friendly way (Macey and Coyne 2005). Thus, FAO has now indicated the use of probiotics as a means of improving the quality of the aquatic environment (Subasinghe *et al.* 2003). Against this background, the aim of the present study was to develop probiotics (Figure 4.2) for the control of *V. anguillarum* and *V. ordalii* infections in rainbow trout.

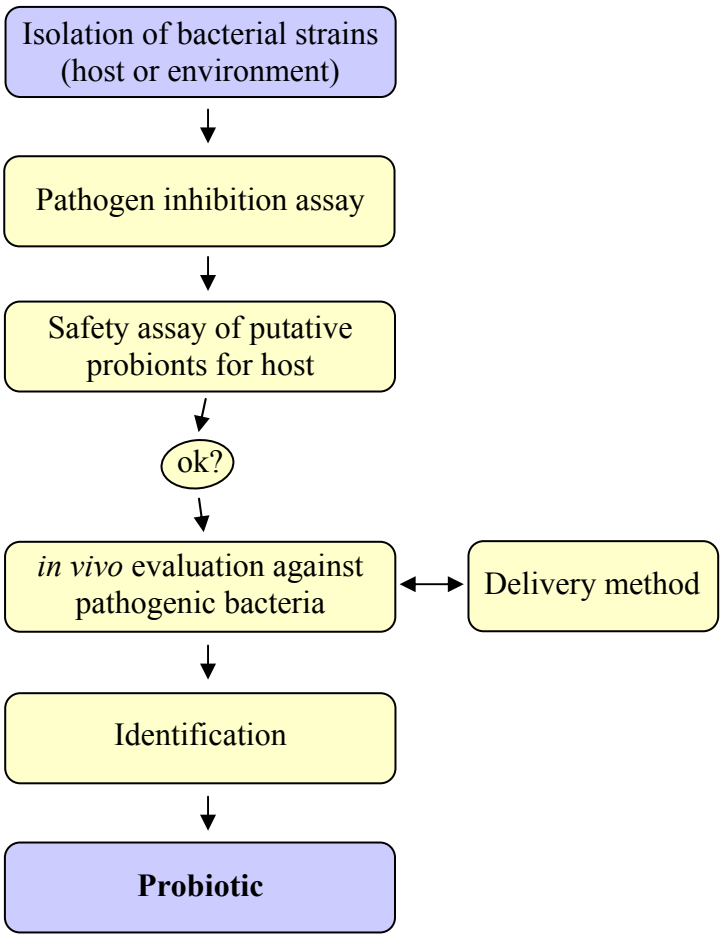

Figure 4.2 Rationale for the development of probiotics, with acknowledgement to Verschuere *et al.* (2000) and Balcázar *et al.* (2006a).

Generally, candidate fish probionts have been obtained from a wide range of sources, including healthy adult fish (Gildberg *et al.* 1997; Gram *et al.* 1999), rearing water (Lauzon *et al.* 2009), fish larvae (Gatesoupe 1999; Ringø and Vadstein 1998), human probiotics *L. rhamnosus* (Nikoskelainen *et al.* 2003) and *L. plantarum* (Picchietti *et al.* 2007) or the gut of chickens (Pan *et al.* 2008) – all of which led to improved health of farmed fish species. However, it is argued that isolation of putative probiotics from the indigenous microbiota of fish or the rearing environment [= assumed to be the natural location of "good" micro-organisms (Gullian *et al.* 2004)] may have the desirable probiotic effect (Verschuere *et al.* 2000). Thus, in this study I have investigated the intestinal microflora of the target host species, i.e. rainbow trout, as a source of potential probiotics.

When isolating the putative probiotics, average viable bacterial counts of $5.4 \pm 7.6 \times 10^6$ cfu g^{-1} were obtained on TSA and this agrees with previous results on microbial counts in rainbow trout intestine (Kim *et al.* 2007). Then, representative colonies were tested for *in vitro* antagonism against *V. anguillarum* and *V. ordalii* by cross streaking, which is a common way for the screening (= elimination of unwanted isolates) and selection of putative probiotics (Austin *et al.* 1995; Verschuere *et al.* 2000; Irianto and Austin 2002a; Brunt and Austin 2005). *In vitro* antagonistic activity is based on the production of inhibitory compounds/substances [= antibiotics or antimicrobial peptides (Sugita *et al.* 1998), organic acids or hydrogen peroxide (Ringø and Gatesoupe 1998)] or on competition for nutrients [= siderophores (Gram and Melchiorsen 1996; Sugita *et al.* 1998)]. Whereas this is an appropriate strategy for beneficial organisms that work by competitive exclusion, it is of course of questionable relevance for other modes of action, such as stimulation of immunity, which was highlighted in this study. Nevertheless, the approach has been successful for the recognition of effective probiotics for a range of fish pathogens (e.g. Irianto and Austin 2002a; Brunt and Austin 2005). Thus by using antagonism as the primary screen, ~19% of the isolates, including SM1 and SM2, demonstrated inhibitory properties against the reference pathogens. Similarly by using the double-layer method of determining antagonism, Riquelme *et al.* (1997) reported ~2% isolates (= 11 out of total 506 bacteria) to be inhibitory against selected pathogens. In addition, extracellular products of five bacteria from a total 106 isolates recovered from the stomach and intestine of common clownfish (*Amphiprion percula*) were inhibitory against *A. hydrophila*, *A. salmonicida*, *V. harveyi*, *V.*

anguillarum, *V. damsela* (= *Photobacterium damselae*), *V. alginolyticus* and *C. piscicola* (Vine *et al.* 2004a). This emphasizes that a small ratio of resident microbiota of fish or the rearing environment can produce various antagonistic compounds and inhibit the growth of pathogens (Robertson *et al.* 2000; Spanggaard *et al.* 2001; Fjellheim *et al.* 2007). In numerous cases, it is these antagonistic bacteria that have been demonstrated to be effective probiotics.

However, it is certainly not definite that *in vitro* antagonism, and the evaluation of other selection methods, i.e. growth characteristics (Vine *et al.* 2004a), and the ability to adhere to the mucus layer (Verschuere *et al.* 2000; Vine *et al.* 2004a; Chabrillón *et al.* 2005a), are necessarily the only suitable characteristics for preselection of probiont candidates for use *in vivo* (Balcázar *et al.* 2006a). As recorded in this study, some strains with desirable *in vitro* characteristics were pathogenic (Riquelme *et al.* 1997) or were unsuccessful in improving the health of the host (Gram *et al.* 2001). For example, putative probiotics selected on their *in vitro* antagonistic ability to *V. anguillarum* were unable to confer protection in rainbow trout (Spanggaard *et al.* 2001). Moreover, Gram *et al.* (2001) demonstrated that results of *in vitro* pathogen inhibition assays could not be used to completely predict an *in vivo* effect since probiotic *Ps. fluorescens* with a strong antagonism against *A. salmonicida* did not protect Atlantic salmon from furunculosis. Therefore, absence or presence of *in vitro* antagonism may not be the criterion for selection (Riquelme *et al.* 1997) or for excluding isolates from further consideration (Rico-Mora *et al.* 1998). Nevertheless, *in vitro* criteria remain necessary for selection of putative probiotics (Verschuere *et al.* 2000; Balcázar *et al.* 2006a) as the approach has the potential to reduce a large collection of isolates to a smaller number, thereby saving expenditure and time.

The next step towards the development of fish probiotics requires examination of any possible harmful effects of the candidate probionts on the host (Verschuere *et al.* 2000; Irianto and Austin 2002a; Brunt and Austin 2005). Thus, pathogenicity by the selected bacterial isolates to the target host should be tested either by injection or by bath (= liquid suspension of putative probiotics) challenge (Verschuere *et al.* 2000). In this study, a total 10 isolates, including SM1 and SM2, were recognized to be harmless to rainbow trout as there was not any sign of any pathological effects or mortalities after challenge via the intraperitoneal (i.p.) or intramuscular (i.m.) routes with the organisms.

This approach followed previous work of Austin *et al.* (1995), Irianto and Austin (2002a) and Brunt and Austin (2005), where fish were challenged i.p. or i.m. with the selected probionts, monitored for 7–14 days, and survivors were sacrificed followed by examination of disease symptoms after inspecting the kidney, spleen and muscle.

Only those bacterial cultures which did not cause any harmful effects were assessed further. In aquaculture, probiotics may be applied either as food supplements [which complies with the definition of a probiotic used by FAO and WHO] or as additives to water (Moriarty 1998) by (i) bathing the host in a bacterial suspension (Austin *et al.* 1995; Gram *et al.* 1999), (ii) addition of the culture directly to rearing water (Moriarty 1998; Spanggaard *et al.* 2001; Gobeli *et al.* 2009), (iii) supplementation with artificial inert diets (Nikoskelainen *et al.* 2001; Aly *et al.* 2008; Vendrell *et al.* 2008), or (iv) via bioencapsulation, i.e. enrichment of live food artemia/rotifers (Gatesoupe 1994; Planas *et al.* 2006). It is evident that most studies have administrated the probiotics to fish or shellfish (larvi)culture by adding them to the diet or directly into the rearing media; the former being more practical than the latter in reality (Hai *et al.* 2009). However, the developmental stage and age of fish and culture environment may be crucial when choosing an appropriate delivery method. This project used a commercially available pelleted diet into which probiotic cultures were mixed and fed to fish in order to determine palatability as well as efficacy. The outcome was that the fish accepted the modified diets prepared with SM1 and SM2, and displayed a better feeding response than the controls. Moreover, the viability of the putative probiotics in the feed was monitored by storing the diet at 4°C and at room temperature. Thus when stored at 4°C for 14 days, the cell viability of SM1 and SM2 was prolonged and fared better compared to storage at room temperature. This points to the benefit of refrigerating the probiotic-containing diet. However, there was a steady decrease in the viability for both probiotics at the two temperatures. In this connection, Aly *et al.* (2008) demonstrated a greater number of viable probiotic cells (i.e. mixtures of *B. subtilis* and *L. acidophilus*, or *B. subtilis* or *L. acidophilus*, alone) and their durability in the feed under refrigeration (4°C) compared with storage at room temperature (25°C). Similarly, Irianto and Austin (2002a), Newaj-Fyzul *et al.* (2007) and Capkin and Altinok (2009) demonstrated higher numbers of viable cells on the feed when stored at 4°C rather than 20–22°C. Interestingly, there was better survival of Gram-positive bacteria than Gram-negative isolates (Irianto and Austin 2002a).

Table 4.1 List of probiotics effective in controlling bacterial diseases of salmonid fish.

Disease / causative agent	Probiont	Host species	Reference
Streptococcosis: *S. iniae*	*A. sobria*, *Bacillus* sp.	rainbow trout	Brunt and Austin (2005), Brunt *et al.* (2007)
Lactococcosis: *La. garvieae*	*A. sobria*, *Bacillus* sp., *Leu. mesenteroides*, *L. plantarum*	rainbow trout	Brunt and Austin (2005), Brunt *et al.* (2007), Vendrell *et al.* (2008)
Furunculosis: *A. salmonicida*	*A. sobria*, *Bacillus* sp., *A. hydrophila*, *Carnobacterium* sp., an unidentified Gram-positive coccus, *V. fluvialis*, *La. lactis*, *Leu. mesenteroides*, *L. sakei*, *L. rhamnosus*, [1]*C. inhibens*	rainbow trout	Brunt *et al.* (2007), Irianto and Austin (2002a), Irianto and Austin (2003), Balcázar *et al.* (2007b), Nikoskelainen *et al.* (2001), Robertson *et al.* (2000)
	[1]*C. inhibens*, *V. alginolyticus*	Atlantic salmon	Robertson *et al.* (2000), Austin *et al.* (1995)
	La. lactis, *Leu. mesenteroides*	brown trout	Balcázar *et al.* (2009)
Vibriosis: *V. anguillarum*	*Ps. fluorescens*, *A. sobria*, *Bacillus* sp., *V. alginolyticus*	rainbow trout, Atlantic salmon	Gram *et al.* (1999), Brunt *et al.* (2007), Austin *et al.* (1995)
V. ordalii	*A. sobria*, *Bacillus* sp., [1]*C. inhibens*, *V. alginolyticus*	rainbow trout, Atlantic salmon	Brunt *et al.* (2007), Robertson *et al.* (2000), Austin *et al.* (1995)
V. harveyi	*A. sobria*	rainbow trout	Arijo *et al.* (2008)
Enteric Redmouth (ERM): *Y. ruckeri*	*A. sobria*, *Bacillus* sp., *B. subtilis* + *B. licheniformis*, [1]*C. inhibens*, *Enterobacter cloacae*, *B. mojavensis*	rainbow trout, Atlantic salmon	Brunt *et al.* (2007), Raida *et al.* (2003), Robertson *et al.* (2000), Capkin and Altinok (2009)
Fin rot: *A. bestiarum*	*A. sobria*, *Brochothrix thermosphacta*	rainbow trout	Pieters *et al.* (2008)
White spot: *Ichthyophthirius multifiliis*	*A. sobria*	rainbow trout	Pieters *et al.* (2008)

A. = *Aeromonas*, *B.* = *Bacillus*, *C.* = *Carnobacterium*, *L.* = *Lactobacillus*, *La.* = *Lactococcus*, *Leu.* = *Leuconostoc*, *Ps.* = *Pseudomonas*, *S.* = *Streptococcus*, *V.* = *Vibrio*.

[1]*Carnobacterium* strain K1 identified as *Carnobacterium inhibens* (Jöborn *et al.* 1999).

In vivo studies to determine the effectiveness of potential probiotics for controlling disease will inevitably involve experimental challenges of the host (Austin *et al.* 1995; Verschuere *et al.* 2000). Thus in this study, juvenile rainbow trout administered with live cells of SM1 and SM2 supplemented diets for 14 days were followed by challenge with *V. anguillarum* or *V. ordalii*, with the results revealing a marked reduction in mortalities when compared to controls. Certainly, the outcome of this study matches previous findings pointing to the greater survival of probiotic-fed fish after experimental challenge with a wide range of bacterial fish pathogens (Aly *et al.* 2008; Vendrell *et al.* 2008), and demonstrated that probiotics are not necessarily specific to a single disease. Similarly, dietary *Bacillus* JB-1 and *A. sobria* GC2 demonstrated effectiveness in controlling multiple bacterial diseases, such as *V. anguillarum*, *V. ordalii*, *La. garvieae*, *A. salmonicida*, *S. iniae* and *Y. ruckeri* in rainbow trout (Brunt *et al.* 2007). Moreover, Robertson *et al.* (2000) confirmed efficacy of *Carnobacterium* sp. at reducing diseases caused by *A. salmonicida*, *V. ordalii* and *Y. ruckeri* in salmonids. Improved resistance to *V. anguillarum* in cod (*G. morhua*) fry was also reported after addition of *C. divergens* (Gildberg and Mikkelsen 1998). The effectiveness of probiotics supplementation against infectious bacterial pathogens of salmonids is summarized in Table 4.1. However, it is noteworthy that a reduction of mortality for certain host-pathogen combinations may not necessarily indicate a universal effect. For example, probiont *Ps. fluorescens*, which was successful in controlling vibriosis in rainbow trout (= vibriosis-rainbow trout system), failed to protect Atlantic salmon against furunculosis, i.e. ineffectual with the furunculosis-salmon system (Gram *et al.* 1999, 2001). As an alternative to viable cells, Brunt and Austin (2005) and Pan *et al.* (2008) observed that the addition of formalized, sonicated, heat-killed and cell-free supernatant of probiotics conferred less protection in rainbow trout and Chinese drum against *S. iniae*, *La. garvieae*, *A. hydrophila* and *V. anguillarum*, which reinforces the benefit of using live cells. Also, Taoka *et al.* (2006b) noted decreased resistance in tilapia (*O. niloticus*) to *E. tarda* infection after oral administration of dead probiotics when compared to the use of live cells. Therefore, the administration of dead/inactivated cells or the supernatant of probiotics does not necessarily reduce bacterial infections, indicating that the maximum benefits of probiotics are mediated in some cases by live bacterial cells.

Subsequently, isolates (i.e. SM1 and SM2) with favourable characteristics, as determined from *in vitro* or *in vivo* selection criteria for probiotic bacteria (Figure 4.2),

were identified. The identification step remains essential since it can provide useful information about the culture requirements and possible virulence gene expression of the selected probiont (Vine *et al.* 2006). Initially, SM1 and SM2 were phenotypically characterized using the API 20E and 20NE system, but test results proved inconclusive since neither of the isolates could be identified. However, by means of diagnostic tables in Cowan and Steel (2003), SM1 and SM2 were tentatively identified as *Micrococcus* sp. (= reclassified to the genus *Kocuria*; Stackebrandt *et al.* 1995) and *Corynebacterium* sp. (= reclassified to the genera of *Rhodococcus*, *Arthrobacter*, *Clavibacter*, or *Curtobacterium*; see Cowan and Steel 2003), respectively. This outcome was verified by partial sequencing of the 16S rDNA gene and the two isolates were identified as *Kocuria* sp. for SM1 and *Rhodococcus* sp. for SM2, respectively. Members of the genus *Kocuria* are characterized as Gram-positive, aerobic, coccoid, nonencapsulated, nonendospore-forming and nonhalophilic organisms (Kim *et al.* 2004; Zhou *et al.* 2008), with many tolerating high salt concentrations (Stackebrandt *et al.* 1995; Kim *et al.* 2004). Their habitats include mammalian skin, soil, rhizoplane, freshwater and marine sediment (Kim *et al.* 2004). The organisms are not considered as primary pathogens of humans and other mammals (Kocur *et al.* 1991). Very little is known about the presence of *Kocuria* in fish apart from the report of Kim *et al.* (2007), who observed the occurrence of some *Kocuria* among the total intestinal bacterial microflora of rainbow trout. However, *Micrococcus* species have been shown to interfere with fish pathogens *in vitro*, such as by the competition for adhesion sites (Chabrillón *et al.* 2005b) and the production of antibiotic substances (El-Rhman *et al.* 2009). Previously, it has been reported that an isolate was successful in controlling *A. salmonicida* infections in rainbow trout (see Irianto and Austin 2002b) and thus was regarded as possessing the suitable criteria to be considered as a potential probiotic. Non-motile Gram-positive actinomycetes of the genus *Rhodococcus* are common in many environmental niches such as soils, rocks, boreholes, groundwater, seawater plants and marine/deep-sea sediments (see Larkin *et al.* 2005; Peng *et al.* 2008). Occurrence of *Rhodococcus* sp. was also reported in fish, namely tilapia (Zhou *et al.* 2009) and trout (Waché *et al.* 2006; Arijo *et al.* 2008). The organisms grow slowly but show persistence in the environment, and some are psychrotrophic in nature (Quek *et al.* 2006). However, rhodococci are of significant environmental and biotechnological importance because certain species have the ability to degrade a large number of organic compounds (= bioremediation), to produce biosurfactants or emulsifiers, to produce exoenzymes and to

survive long-term under extremely harsh conditions (Larkin *et al.* 2005; Das *et al.* 2008; Quatrini *et al.* 2008; Peng *et al.* 2008). Moreover, actinomycetes are reported to produce antibacterial substances, and the antibiotic product administered to the tiger shrimp (*Penaeus monodon*) post larvae (PL–20) as feed supplement reduced infection due to white spot syndrome virus (Kumar *et al.* 2006). Therefore, there is a potential role in aquaculture as probiotics, i.e. association in nutrient cycles (= water quality improvement) of aquaculture ponds, competitive exclusion of pathogens and host digestion processes.

The dose of probiotics, which is the cell concentration of probiotic (i.e. no. of probiotic cells mL^{-1}) available to the aquatic host (Vine *et al.* 2006) used in this study corresponded well with previous work with probiotics (e.g. Nikoskelainen *et al.* 2003; Kim and Austin 2006a). Thus, considering different dose levels (= 10^5–10^9 cells g^{-1} feed), it was revealed that any dose below or above 10^7 and 10^8 cells g^{-1} for SM1 and SM2, respectively, did not result in good levels of protection. Similarly, Newaj-Fyzul *et al.* (2007) demonstrated that doses of *B. subtilis* lower and higher than 10^7 cells g^{-1} of feed were less successful at enhancing resistance to *Aeromonas* infection in rainbow trout. Moreover, feeding of rainbow trout with *L. rhamnosus*-supplemented diets at 10^9 cfu g^{-1} for 51 days led to reduced mortality rates from 52.6% in the control to 18.9% when compared to mortality rates of 46.3% for the dose 10^{12} cfu g^{-1}, following challenge with *A. salmonicida* (Nikoskelainen *et al.* 2001). Gill and Rutherfurd (2001) observed a dose-response effect on the immune function of mice fed *L. rhamnosus* supplemented milk. For example, a dose of 10^9 cells of *L. rhamnosus* day^{-1}, which was required to significantly enhance the phagocytic capacity of peritoneal cells compared to control mice, was increased to 10^{11} day^{-1} and this did not further increase peritoneal cell phagocytosis. In contrast, a significant incremental enhancement in the phagocytic capacity of blood leucocytes was found by increasing the dose from 10^7 to 10^{11} cells day^{-1}. Therefore, a dose-effect relationship should be carefully determined to avoid overdosing with resultant lower efficacy and unnecessary costs, or conversely underdosing, which reduces the efficiency of the probiont (Vine *et al.* 2006).

Equally, consideration of the length of feeding time holds fundamental importance while developing probiotics. Studies with probiotics to date have involved the use of different feeding durations, for example 1–8 week feeding regimes, leading to improved disease resistance in farmed fish (Gildberg *et al.* 1997; Robertson *et al.* 2000; Irianto

and Austin 2002a; Raida *et al.* 2003; Aly *et al.* 2008; Pan *et al.* 2008; Vendrell *et al.* 2008), but the basis for choosing these periods is often unclear. Therefore, in this study, fish were fed a diet containing live cells of SM1 for up to four weeks with weekly challenges to determine the optimal feeding duration for probiotics maximizing the host protection. The results revealed that a two-week feeding regime with SM1 led to higher disease protection in rainbow trout against *V. anguillarum* than any of the lower or higher doses. Indeed, supplementation of *Bacillus* or *A. sobria* at 2×10^8 cells g^{-1} feed for two weeks conferred protection against *V. anguillarum*, *V. ordalii*, *La. garvieae*, *A. salmonicida*, *S. iniae* and *Y. ruckeri* in rainbow trout (Brunt *et al.* 2007). Furthermore, it has been demonstrated in the same fish species that the addition of *Leu. mesenteroides* and *L. plantarum* at 10^7 cfu g^{-1} for 30 days (Vendrell *et al.* 2008), and a mixture of *B. subtilis* and *B. licheniformis* at 4×10^4 spores g^{-1} for 42 days (Raida *et al.* 2003) controlled infections caused by *La. garvieae* and *Y. ruckeri*. Also, dietary *L. plantarum* in grouper (Son *et al.* 2009), *C. butyricum* in Chinese drum (Pan *et al.* 2008), and *B. subtilis*, *L. acidophilus* or a mixture of both in tilapia (Aly *et al.* 2008) were beneficial when used for 28, 30 and 60 days, respectively, reducing mortality following challenge with *Streptococcus* sp., *V. anguillarum*, *A. hydrophila*, *Ps. fluorescens* and *S. iniae*. Conversely, one-week administration of SM1 significantly reduced the fish survival after challenge with the pathogen counter to other feeding regimes, which corroborates well with some previous findings (e.g. Robertson *et al.* 2000). Therefore, it is apparent that feeding with the probiotics for two or more weeks is effective in controlling fish diseases. In conclusion, a two-week feeding regime with SM1 leads to the best defence against *V. anguillarum* in rainbow trout.

The combination of either multistrain or multispecies formulations may improve the efficacy of probiotics by triggering synergistic beneficial effects on the health of the host, i.e. improvement or prolongation of the desirable effects (Timmerman *et al.* 2004). Although, an equi-mixture of SM1 and SM2 significantly enhanced fish survival against *V. anguillarum* or *V. ordalii* infections in comparison with the controls, the beneficial effect was not statistically different when compared to the application of single cultures of probiotic. Similarly, Irianto and Austin (2002a) did not reveal further benefit by using an equi-mixture of *A. hydrophila*, *V. fluvialis*, *Carnobacterium* sp. and a Gram-positive coccus A1-6, over mono species preparations of probiotic in controlling *A. salmonicida* in rainbow trout. These findings disagree with Aly *et al.* (2008), who noted

significantly higher protection in tilapia against several pathogens (= *A. hydrophila*, *Ps. fluorescens* and *S. iniae*) when fed mixtures of *B. subtilis* and *L. acidophilus* for one month compared with the groups that received either *B. subtilis* or *L. acidophilus* alone. In contrast, tilapia administered with a mixture of *M. luteus* and *Pseudomonas* sp. for 90 days did not resist *A. hydrophila* infection, reaching mortalities to 80% compared with 25% mortalities when fed a single culture of *M. luteus* (El-Rhman *et al.* 2009). These data suggest that the level of host protection due to viable probiotics may differ in relation to the fish species, and the type of probiotic strain and their appropriate combinations.

It is argued that the fish immune system lacks memory (see Ortuño *et al.* 2002), and as such the duration of probiotic induced beneficial responses, which is primarily mediated by innate immunity (Brunt and Austin 2005; Kim and Austin 2006a; Brunt *et al.* 2007) may inevitably be shorter than that of the specific immune responses, e.g. the protective immunity conferred from vaccination. Therefore, the duration of disease protection in rainbow trout after feeding with probiotics was determined by examining the long term beneficial effect of probiotics. For this, following a 14 day feeding regime with probiotic SM1, rainbow trout were challenged with *V. anguillarum* at weekly intervals for 5-weeks with the data revealing a significantly lower mortality for up to four weeks compared with the untreated controls. These results illustrate a carry-over effect of probiotics. This is relevant because in previous work adult rainbow trout orally vaccinated with attenuated viral haemorrhagic septicaemia virus (VHSV) strain ATT 150 were protected significantly upon challenge with the virulent VHSA strain Fi13, six weeks after vaccination (Adelmann *et al.* 2008). Moreover, four weeks after immunization, mice and soft-shelled turtle (*Trionyx sinensis*) orally administered with alginate microparticle *A. sobria* vaccine had significantly higher protective immunity against *A. sobria* (Sun *et al.* 2007; Yang *et al.* 2007), and rainbow trout (weight = 22 g) showed a RPS value of 50% against lactococcosis when receiving oral immunization with *La. garvieae* cells encapsulated in alginate-acetone capsules (Romalde *et al.* 2004). Analogous results were also reported by Kwon *et al.* (2007), who observed a higher survival of juvenile olive flounder (*Paralichthys olivaceus*) against *E. tarda* infection, two weeks after dietary supplementation of *E. tarda* ghosts or formalin-killed *E. tarda*. Clearly this study has highlighted the fact that the beneficial effect of dietary SM1 to the

defense mechanisms of rainbow trout extends beyond the withdrawal period of probiotic feeding.

Thus far, the administration of dead/inactivated cells or the supernatant of probiotics did not necessarily reduce bacterial infections in fish, which reinforced the benefit of using live cells (Brunt and Austin 2005; Taoka *et al.* 2006b; Pan *et al.* 2008). In this context, the viable cells of two probiotics SM1 and SM2 were highly effective in controlling vibriosis in rainbow trout. To move forward with the understanding of probiotics, this study investigated the efficacy of sub-cellular components of probiotics to determine which part of the cell is responsible in conferring host protection. Thus, the use of cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2 led to significant resistance to *V. anguillarum* infection in rainbow trout. Conversely, extracellular proteins (ECPs) of both probiotics fared less well. From previous work, cell-free supernatants of probiotics offered less protection compared to viable cells (Brunt and Austin 2005; Pan *et al.* 2008). Moreover, immunization with membrane vesicle (MVs) rich supernatant of *Flavobacterium psychrophilum* cells did not protect rainbow trout against *F. psychrophilum* infection (Aoki *et al.* 2007). Conversely, in a recent study, Abbass *et al.* (2010) observed that subcellular components, i.e. CWPs, WCPs, lipopolysaccharides (LPS) and outer membrane proteins (OMPs) of the probiotics *A. sobria* and *B. subtilis*, when administered to rainbow trout, led to 100% survival compared with 10% survival in the controls against a new biogroup of *Y. ruckeri* that has been resistant to conventional vaccines. Moreover, injection with OMPs of *A. hydrophila* was shown to control infection in goldfish caused by pathogenic bacteria (Rahman and Kawai 2000). Overall, this study pointed to the potential of using cellular components of probiotics in controlling bacterial fish diseases and may well explain the parts of the cells involved in protection.

The fundamental question is how dietary probiotics may induce disease resistance in rainbow trout. The answer is not conclusive since an array of possible modes of action have been suggested, which might have complex interrelationships among themselves. It is apparent that the potential effects of probiotics in fish include competitive exclusion (i.e. production of inhibitory compounds, competition for chemicals, oxygen or available energy, and competition for adhesion sites), inhibition of virulence gene expression or disruption of quorum sensing molecules, improvement of water quality,

immunostimulation, a source of macro and/or micronutrients, improvement in the microbial balance, and enzymatic contribution to digestion (Vershuere *et al*. 2000; Irianto and Austin 2002b; Balcázar *et al.* 2006a; Gómez and Balcázar 2008; Tinh *et al*. 2008; Hill *et al.* 2009; Picchietti *et al.* 2009).

In vitro studies showed that *Kocuria* SM1 and *Rhodococcus* SM2 were inhibitory against *V. anguillarum* and *V. ordalii*, indicating both probiotics secrete inhibitory compounds. Therefore, it cannot be ruled out that there is the possibility of out-competing pathogens by antibiosis via the production of low molecular weight inhibitors *in vivo*. Some probiotic micro-organisms were capable of preventing proliferation of pathogens on intestinal cells by competing for attachment sites (Mukai *et al.* 2002; Chabrillón *et al.* 2005a; Gueimonde *et al.* 2006). In agreement with these results, Balcázar *et al.* (2008) demonstrated that adhesion of the fish pathogens *A. hydrophila*, *A. salmonicida*, *V. anguillarum* and *Y. ruckeri* to intestinal mucus of rainbow trout was reduced by lactic acid bacteria *La. lactis*, *L. plantarum* and *L. fermentum*, respectively, in *in vitro* conditions. This anti-adhesion activity may be explained by the secretion of antimicrobial substances, such as antibiotics or siderophores, by these bacteria (Mukai *et al.* 2002; Balcázar *et al.* 2008). However, neither SM1 nor SM2 produced siderophores.

Likewise, it has been stated that probiotic bacteria should possess the characteristics of gastrointestinal viability, i.e. the capacity of entry and survival during transit through the stomach and then persistence in the intestinal tract in order to provide beneficial effects for the host (Chou and Weimer 1999; Huang and Adams 2004). Acid concentration (pH) in the fish stomach ranges from 2–4, then conditions become alkaline (pH 7–9) immediately below the pylorus, dropping a little to a maximum of 8.6 in the upper intestine, and finally approaching neutrality in the hindgut (Smith 1980). This suggests that probiotic cultures upon oral administration have to overcome major physiological challenges due to the barrier of extremely low pH of the stomach and the antimicrobial action of pepsin (Huang and Adams 2004). In this scenario, *in vitro* acid tolerance ability of the probiotics was tested, and the results indicated that both SM1 and SM2 were able to grow at pH 2–11. Even the addition of pepsin (3 mg mL^{-1}) at pH 2 or 3 did not affect the viability of the isolates. Besides, SM1 and SM2 survived well when exposed to pancreatin (1 mg mL^{-1}) at pH 8. Although the pH of the small

intestine (i.e. 7.0–8.5) is more favourable for bacterial survival, the presence of pancreatin or bile salts can have unfavourable effects (see Hosseini *et al.* 2009). However, the two probiotics had the capacity to withstand the effects of pepsin during gastric transit, also being resistant to the action of pancreatin in the small intestine. Certainly, these results are in agreement with those reported by Balcázar *et al.* (2008) for probiotics *La. lactis*, *L. plantarum* and *L. fermentum* that showed high resistance to pH values from 2.5 to 6.5, but lost their viability at pH 1–2, although *L. plantarum* tolerated pH 2 slightly. In contrast, Kim and Austin (2008) observed growth of probiotics ***C. maltaromaticum*** and ***C. divergens***, derived from rainbow trout intestine, at pH 5–10. So, depending on the tolerance capacity to stomach acids and bile salts, the survivability of probiotic bacteria in the digestive tract is not surprising. For example, Kim and Austin (2006a) recovered ***C. maltaromaticum*** and ***C. divergens*** from the digestive tract of rainbow trout after 14 days feeding with diets supplemented with these bacteria. Similar observations have been made previously with other probiotic strains (i.e. *A. hydrophila*, *V. fluvialis*, *V. alginolyticus*, *C. inhibens*, *B. subtilis*) fed to fish (Robertson *et al*. 2000; Irianto and Austin 2002a; Newaj-Fyzul *et al.* 2007). However, it is argued that these probiotic cultures do not show spontaneous primary colonization in the digestive tract, instead sustaining a transient state as long as the bacteria are introduced via the feed, and then seem to disappear upon switching to regular feed (Robertson *et al*. 2000; Newaj-Fyzul *et al.* 2007).

Another promising side of using dietary probiotics is the potential improvement in feed efficiency, which may be reflected through better growth rate of the animal and improved feed utilization. In the present study, an improvement of SGR, FCR and PER was observed in fish fed with SM2 compared to the control group, although the result was statistically indifferent. In contrast, improvements were not recorded when using SM1. In this connection, Sáenz de Rodrigáñez *et al.* (2009) reported significantly higher growth and nutrient utilization in juvenile sole (*Solea senegalensis* weight = 10–15 g) after dietary supplementation with probiotics (= *Alteromonadaceae* family) for 60 days. Similar results have been reported in African catfish (*Clarias gariepinus*) fingerlings maintained on diet supplemented with *L. acidophilus* for 84 days (Al-Dohail *et al.* 2009), and in larval sea bass (*D. labrax*) administered with *L. delbrueckii delbrueckii* via rotifers or *Artemia* nauplii between days 11 and 70 posthatch (Carnevali *et al.* 2006) than those fed diets without probiotic. Moreover, a significant improvement of FCR,

SGR and PER was observed in rainbow trout (weight = ~45 g), previously treated with antibiotic, after 70 days feeding on mixed *B. subtilis* and *B. licheniformis* supplemented diets (Merrifield *et al.* 2010b). Surprisingly in a similar experiment keeping most of the parameters identical, Merrifield *et al.* (2010a) observed no statistical improvements of SGR in rainbow trout (weight = ~70 g) fed with probiotics, although an enhanced SGR was noted compared to the controls. It was proposed that, as in terrestrial animals, some probiotic strains may be a source of micro- and macroelements in feed (Verschuere *et al.* 2000) and their activity in the digestive tract may stimulate the specific and/or total activities of digestive enzymes, such as amylase, protease and lipase activities (Balcázar *et al.* 2006a; see Sáenz de Rodrigáñez *et al.* 2009) thereby improving the whole digestive process, and enhancing the digestibility of feed and the effective utilization of nutritive supplies. However, in this study, the overall digestive enzyme activities of rainbow trout were not modified after probiotic treatment and the levels were virtually analogous to the controls. Interestingly, SM2 demonstrated slightly better extracellular enzymatic activities (i.e. carbohydrase, ester hydrolases, phosphohydrolases and protease) compared with SM1, but it was not clear whether these enzymes led to better growth in fish treated with SM2 in comparison to other treatments.

The organosomatic indices, i.e. hepatosomatic index and splenosomatic index are indicators of the health status of fish (see Abd-El-Rhman 2009). Fish spleens are macrophage-rich tissues, and a key source of antibody production and immunological memory (Hadidi *et al.* 2008). Thus, rainbow trout with greater spleen weights were significantly more resistant to *F. psychrophilum* (= aetiological agent of bacterial cold-water disease and rainbow trout fry syndrome) than those of the susceptible fish, although the result was pathogen-specific (Hadidi *et al.* 2008). This suggests a positive correlation between fish survival and spleen size when infected with potential diseases. However, the hepatosomatic and splenosomatic indices remained unaffected by any treatment in the present study. It should be emphasized that the outcome of probiotic use is not always reproducible. There may be variability of results noted even after using the same probiotic with the same type of fish (see Panigrahi *et al.* 2004 in comparison with Panigrahi *et al.* 2005, and Merrifield *et al.* 2010a in comparison with Merrifield *et al.* 2010b).

Irianto and Austin (2002a) provided evidence that probiotics act by stimulating the immune response in fish. This particular role of probiotics corroborates well to the

present study where innate immune parameters were enhanced in fish treated with SM1 or SM2 compared to the controls. Certainly, immunostimulation has been identified as the dominant mode of action for a range of probiotics, including representatives of both Gram-positive and Gram-negative bacterial taxa (Austin and Austin 2007). In agreement with previous studies, the use of SM1 or SM2 led to higher leucocyte (Brunt and Austin 2005; Newaj-Fyzul *et al.* 2007) and erythrocyte (Brunt and Austin 2005) counts in the blood, although statistical significance in the data was not always reported (Brunt *et al.* 2007). It was observed that the presence of high blood leucocyte numbers was accompanied by an enhanced oxidative burst and phagocytic activity in yellowtail and rainbow trout (see Gannam and Schrock 2001), which have been associated with bacteriocidal activity and functioning as a defensive immune mechanism (Claver and Quaglia 2009). The leukocytes usually develop from an enhanced ability of a particular cell/tissue to counter infection (Cruickshank 1965), and are recognized as vital immune competent cells (e.g. lymphocytes and monocytes) mediating specific (= acquired or adaptive immunity) and non-specific immunity in fish (Wang *et al.* 2006). However, erythrocytes in fish can equally act as immune cells, binding and engulfing *Candida albicans* (Passantino *et al.* 2002). So, probiotics can enhance the number of erythrocytes, granulocytes, macrophages and lymphocytes in fish (Irianto and Austin 2002a; Kumar *et al.* 2008).

Phagocytic activity is responsible for the generation of inflammatory responses before antibody production. Moreover, phagocytic activity results in an additional recruitment of immunocompetent cells to the inflammatory site, thus playing a crucial role in antibacterial host defenses (Isolauri *et al.* 2001; Nayak 2010). In agreement with previous studies using rainbow trout, feeding with viable probiotics for two weeks or more leads to higher macrophage phagocytosis (Brunt and Austin 2005; Panigrahi *et al.* 2005; Pieters *et al.* 2008), which is a mechanism involved in the eventual killing of phagocytosed infectious agents and also serves as an innate immune effector (Greenberg and Grinstein 2002). A similar beneficial effect had already been observed on the phagocytic activity of finfish fed with *L. plantarum* at $10^{6–10}$ cfu kg^{-1} (Son *et al.* 2009) yeast *Debaryomyces hansenii* at 10^{6} cfu g^{-1} (Reyes-Becerril *et al.* 2008), *C. butyricum* at 10^{8} cells g^{-1} (Pan *et al.* 2008), and *L. delbrüeckii* subsp. *lactis* and *B. subtilis* at 10^{7} cfu g^{-1} or their equi-mixtures (Salinas *et al.* 2008) for between two and

four weeks. Conversely, *La. lactis* was unsuccessful in enhancing the phagocytic activity of head kidney macrophages in turbot (Villamil *et al.* 2002).

As in other vertebrates after activation, fish phagocytic cells, i.e. monocytes/macrophages and neutrophils, are able to generate superoxide anion (O_2^-), which is a measure of respiratory burst activity, hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) and hydroxyl radical (OH^-), all of which are highly microbiocidal (Secombes 1996; Ellis 2001; Neumann *et al.* 2001) and can be associated with the extracellular killing of pathogens (Hardie *et al.* 1996; Secombes 1996; Itou *et al.* 1997). Data suggest that increases in the oxidative killing mechanism, as observed with blood/macrophage superoxide anion and macrophage peroxidase activities in rainbow trout fed SM1, SM2 or their equi-mixture, has been correlated with enhanced pathogen killing capacity of phagocytes in fish (Jørgensen *et al.* 1993; Sharp and Secombes 1993) and so improvement in disease resistance is likely. The present results are in accordance with those performed in fish where respiratory burst (Nikoskelainen *et al.* 2003; Brunt and Austin 2005; Aly et al. 2008) or peroxidase (Rodríguez *et al.* 2003; Reyes-Becerril *et al.* 2008; Salinas *et al.* 2008) activities were enhanced by dietary supplementation with probiotic bacteria and yeast for two and four weeks. Moreover, rainbow trout fed with other probiotics, i.e. *A. sobria* (Brunt *et al.* 2007) and *C. divergens* (Kim and Austin 2006a), demonstrated stimulation of the respiratory burst activity of macrophages after 2 week feeding regimes. Similar to these results, fingerlings of rohu (*L. rohita*) with an average weight of 15 ± 2 g demonstrated significantly higher respiratory burst activity compared to the controls when fed for 2 weeks with *B. subtilis* (Kumar *et al.* 2008). However, the corresponding increase in the activity of serum peroxidase, as recorded here, was not surprising since phagocytes produce reactive oxygen species (ROS) during respiratory burst (Neumann *et al.* 2001), which is believed to exert oxidative stress to the fish and thus requires to be removed (Newaj-Fyzul *et al.* 2007). Evidently, the peroxidase content in the serum of gilthead sea bream and rainbow trout was significantly higher when they were fed with *Bacillus* or a mixture of *Lactobacillus* and *Bacillus* (Salinas *et al.* 2008), and *B. subtilis* (Newaj-Fyzul *et al.* 2007) supplemented diets. Likewise, *Enterococcus faecium* (1×10^7 cfu mL^{-1}) was reported to elevate the serum peroxidase content in tilapia when added to rearing water every 4 days for 40 days (Wang *et al.* 2008). Conversely, Reyes-Becerril *et al.* (2008) did not observe any significant increase

in peroxidase activity of gilthead sea bream fed with diets containing live yeast *D. hansenii*, although a higher activity was noted.

The elevated bacterial killing activity of HK macrophages as observed in this study is in agreement with another report using rainbow trout, which were fed with a probiotic, *B. subtilis* (Newaj-Fyzul *et al.* 2007). Moreover in agreement with this study, tilapia treated with *B. subtilis*, *L. acidophilus* or their mixture led to a significantly higher serum bacteriocidal activity against pathogens *A. hydrophila, Ps. fluorescens* and *S. iniae*, than in the untreated control group (Aly *et al.* 2008). Similar views were put forward by Kumar *et al.* (2008), who noted a significant increase of serum bacteriocidal activity in rohu fed with *B. subtilis* in comparison to the control group. Furthermore, plasma bacteriocidal activity in tilapia was enhanced by commercial probiotics either introduced as live or dead cells via the oral route, or as viable cells via rearing water (Taoka *et al.* 2006b). Conversely, the bacteriocidal activity of serum was not modified by use of *La. lactis* in turbot (Villamil *et al.* 2002).

In teleosts, the complement system plays a role in the adaptive immune response as well as being involved in chemotaxis, opsonization, phagocytosis and degradation of pathogens (Swain and Nayak 2009). Therefore, the alternative complement activity, which is an antibody-independent pathway with higher levels in fish serum compared to mammalian serum (Yano 1992), may be directly involved in the lysis of micro-organisms (Ellis 1999). Here, the differences in complement levels were statistically significant when compared with the controls, and support the findings of previous studies involving probiotics and a range of fish species (Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2004; Balcázar *et al.* 2007a). It is noteworthy that heat inactivated probiotics Pdp11 or 51M6 can also stimulate complement components in fish (Choi and Yoon 2008).

Protection of a host against the onslaught of pathogens may reflect increased serum lysozyme activity. Lysozyme, which is a key humoral innate defence parameter (i.e. bacteriocidal enzyme) in invertebrates and vertebrates (Magnadóttir *et al.* 2005), can hydrolyse peptidoglycan in bacterial cell walls, thereby causing lysis of mainly Gram-positive bacterial species, and in conjunction with complement, even some Gram-negative taxa (Ellis 1990; Alexander and Ingram 1992). Stimulation of lysozyme

activity has been recognized after two, four and six weeks of feeding with *C. divergens* and *L. rhamnosus* in rainbow trout (Panigrahi *et al.* 2005; Kim and Austin 2006a), *C. butyricum* in Chinese drum (Pan *et al.* 2008), *S. cerevisiae* in sea bream (Rodríguez *et al.* 2003), and *L. plantarum* in grouper (Son *et al.* 2009), respectively. Increases in serum lysozyme have been recognized for *B. subtilis* and *L. acidophilus* in tilapia (Aly *et al.* 2008), and for *La. lactis* subsp. *lactis*, *Leu. mesenteroides* and *L. sakei* in brown trout (Balcázar *et al.* 2007a) as feed additives. Possibly, such enhanced nonspecific factors of the immune system may have provided defence against infection by the pathogen. However, some studies failed to detect any specific change in serum lysozyme level after dietary supplementation of probiotics, namely *L. sakei*, *La. lactis* subsp. *lactis*, *Leu. mesenteroides*, *L. rhamnosus* and *A. sobria* in fish (Panigrahi *et al.* 2005; Balcázar *et al.* 2007a,b; Brunt *et al.* 2007).

Immunoglobulins, principally immunoglobulin M (IgM), are major components of the teleost humoral immune system, and are found both in soluble (= secreted) and membrane-bound forms (Cuesta *et al.* 2004). The soluble form, which occurs in blood and other fluids, is secreted from B cells, and is regarded as an immune effector molecule (Ross *et al.* 1998). As noted in this study, elevation of immunoglobulin level after dietary supplementation of probiotics has been reported in many fish species (Panigrahi *et al.* 2004; Nayak *et al.* 2007; Al-Dohail *et al.* 2009). Moreover, 10–30 days administration of viable and non-viable forms of *L. rhamnosus* elevated plasma immunoglobulin levels in rainbow trout (Panigrahi *et al.* 2005). Even one week supplementation of *L. rhamnosus* at a dose of 2.8×10^8 cfu g^{-1} feed was found to significantly increase the immunoglobulin level in rainbow trout (Nikoskelainen *et al.* 2003). Although not statistically significant, Balcázar *et al.* (2007a) observed an increase in immunoglobulin level in brown trout after feeding *La. lactis* subsp. *lactis*, *L. sakei* and *Leu. mesenteroides* supplemented 10^6 cfu g^{-1} feed for a period of 2–4 weeks.

The antiprotease activities in serum and other body fluids, principally α_1-antiprotease, α_2-antiplasmin and α_2-macroglobulin, are antienzymes and are characterized by their capacity to delay or inhibit pathogens that attack through proteolytic enzyme secretion (Ellis 2001; Magnadóttir 2006). These activities are generally high, and appear to be hardly modulated in fish even by immunization or infection (Magnadóttir 2006). In this study, the total antiprotease activity as measured by the mean antitrypsin activity of sera

was elevated in the probiotic-fed fish as compared with the controls, with the differences being significant at week two. Similarly, it was reported that higher levels of total, α_1 and α_2-antiprotease occurred in the serum of rainbow trout after feeding with probiotics (Brunt *et al.* 2007; Newaj-Fyzul *et al.* 2007). However, a 10-fold difference in α_2-macroglobulin activity between rainbow trout and brook trout has been correlated with their differing resistance to the pathogenicity of *A. salmonicida* (Freedman 1991).

Certainly in this study, a two-week feeding regime with SM1 leads to the best defence against *V. anguillarum* in rainbow trout, with protection linked to significant stimulation of macrophage phagocytic activity and serum total protein levels as compared to other treatments and controls. Moreover, in comparison with the controls, serum antiprotease and lysozyme activities were significantly enhanced at week two, but the differences became non-significant by weeks three and four of the experiment. Likewise, macrophage peroxidase content was pronounced at the end of week two. The facts suggest that an elevated immunomodulatory effect after two weeks of probiotic feeding was the key determinant in maximizing host resistance to disease. Similarly, maximum stimulation of macrophage phagocytosis (Rodríguez *et al.* 2003; Salinas *et al.* 2005; Son *et al.* 2009), peroxidase activity (Rodríguez *et al.* 2003; Salinas *et al.* 2005), and serum lysozyme (Panigrahin *et al.* 2005) level has been recorded after 10–14 days dietary supplementation of probiotics in fish involving other feeding regimes. Overall, this study reinforced the importance of probiotics in stimulating the innate immune response of rainbow trout. This contrasts with the previous accepted notion that probiotics act by competitive exclusion, which may include competition for space and nutrients in the digestive tract, or the localized production of antibiotics or low molecular weight inhibitors (Verschuere *et al.* 2000; Irianto and Austin 2002b; Balcázar *et al.* 2006a).

Following withdrawal of dietary SM1, this study found the enhancement of several innate immune parameters in rainbow trout for up to 5 weeks compared to those of the controls, although statistical significance in the data was not always noted at different time points in the experimental groups. Of relevance, the administration of *La. lactis* subsp. *lactis* and *Leu. mesenteroides* at 10^6 cfu g^{-1} into the diet of brown trout has been reported to significantly enhance the serum lysozyme activity, including an increase in the levels of serum alternative complement activity and plasma total immunoglobulin

between 1–2 weeks of post-probiotic feeding (Balcázar *et al.* 2007a). In a similar study, Nikoskelainen *et al.* (2003) observed stimulation in the respiratory burst and complement bacteriocidal activity at 1 week after cessation of feeding of 2.8×10^8 cfu g^{-1} *L. rhamnosus* to rainbow trout; but there was a significant elevation of the natural antibody (= serum immunoglobulin) level in relation to nonstimulated fish at 2 weeks. Comparable results were also obtained by Panigrahi *et al.* (2005), who demonstrated non-significant improvement of phagocytosis, lysozyme and total immunoglobulin levels in rainbow trout 15 days after withdrawal of viable *L. rhamnosus* cells from the diet. However, in this study, the level of the immune response was decreased over time; principally serum lysozyme dropped significantly at the end of the experiment; a phenomenon which is in agreement with findings of other workers (Nikoskelainen *et al.* 2003; Balcázar *et al.* 2007a). Certainly, these observations indicate that there may be an innate memory (= long-term protection) that depends on the steady maintenance of immune response from the induction phase, which might explain the longevity of the disease resistance scenario in rainbow trout in the present study. Undoubtedly, the proliferation rate and populations of lymphocytes produced may be the key for the magnitude and duration of immunity against infections (Eggset *et al.* 1997) suggesting the fundamental importance of persistence of an immune activator in the support of longer immunity.

Nevertheless, in this particular study, rainbow trout fed with standard commercial diet, after previous priming with dietary SM1, were significantly protected against *V. anguillarum* for 4 weeks and produced immune responses possibly comparable to those demonstrated by vaccine. Clearly, fish vaccinology works through stimulation of memory cells, including modulation of innate defense mechanisms (Kozinska and Guz 2004; Robertson *et al.* 2005) and usually provides long-term protection against specific pathogens (Eggset *et al.* 1997). Various recent publications reported that probiotics, like vaccines, led to the induction of a variety of cytokines (= cellular immunity) such as interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β (Kim and Austin 2006b; Panigrahi *et al.* 2007), and lead to an increase in the memory T cell population (Picchietti *et al.* 2009) in naïve fish or fish exposed previously to probiotic-supplemented diet. Cytokines are stimulators and effectors of all immune and inflammatory responses (Kelso 1998), and may be involved in the continued existence of memory cells with the help of interferon α (type I interferon) and

IL-15 (see Zanetti and Croft 2001). Therefore, it may be hypothesized that the mode of action of probiotics is perhaps comparable to oral vaccines (Irianto and Austin 2002b) chiefly due to stimulation of cellular immunity (Irianto and Austin 2002a). However, immunological memory is a functional event and is supposed to be a property of the overall immune system, but not solely through memory cells, i.e. B (= humoral immunity) and T (= cellular immunity) cells (Zanetti and Croft 2001). The possibility of stimulation of the cytokine genes involved in the immune response after probiotic feeding remains a matter of further investigation.

Cell components of micro-organisms have been reported to activate the immune system of many animals, including fish. For example, LPS, a component of the outer cell membrane of Gram-negative bacteria, possesses immunogenic properties, and a small dose can induce the production/activation of antibody, lysozyme, alternative complement pathway, B and T lymphocytes, cytokines like IL-2 and -6, pro-inflammatory cytokines like IL-1β, TNF-α and several other factors from macrophages, including phagocytic activity in fish (Nayak *et al.* 2008; Nya and Austin 2010). OMPs of Gram-negative bacteria are also known to be immunodominant antigens, and may provoke strong humoral and cellular immune responses in fish (Boesen *et al.* 1997; Arijo *et al.* 2004). Moreover after inoculation, ECPs may often lead to adequate immunity against piscine pathogens, and some authors has suggested the importance of including inactivated ECPs in the design of effective vaccines (Collado *et al.* 2000; Zorrilla *et al.* 2003; LaFrentz *et al.* 2004). β-glucans, which are polysaccharides from the cell walls of yeast and fungi, are also found in plants, algae and some bacteria, and have been determined to enhance specific and non-specific immune responses in several fish species, i.e. yellow croaker, Asian catfish, carp and zebrafish (Misra *et al.* 2006; El-Boshy *et al.* 2010). Furthermore, nucleotides and RNA have been shown to have immunostimulatory effects, and thus enhance fish resistance to pathogens (Li *et al.* 2004). Similarly, bacterial DNA is reported to activate antigen-presenting cells (APCs), in mice and fish models (see Hong *et al.* 2006). These data suggest that the non-specific defence of vertebrates has evolved towards recognition of conserved microbial structures, i.e. yeast/fungal cell wall β-glucan, bacterial LPS and peptidoglycan, bacterial DNA and viral double-stranded RNA – all of which have been reported to enhance the host resistance against microbial diseases (Robertsen 1999). Therefore, in

this study, a protective immune response in rainbow trout after inoculation with subcellular components of probiotics was not surprising.

Overall, it is argued that the immunomodulatory effects of probiotics in fish are still poorly understood albeit that a number of previous studies have assessed systemic immune responses *in vivo* (Irianto and Austin 2002a; Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2004, 2005, 2007; Kim and Austin 2006a,b; Brunt *et al.* 2007; Aly *et al.* 2008; Arijo *et al.* 2008; Pieters *et al.* 2008). It is suggested that the interactions of probiotics and/or their components/products (= antigenic particles) with gut epithelial cells, gut immune system [= gut associated lymphoid tissue (GALT)] and gastrointestinal microbiota might control the mechanism behind the immunostimulatory activity (Tinh *et al.* 2008; Nayak 2010). For example, the effect of probiotics in stimulating the gut immune system is documented in some fish species, with a noticeable increase in the number of Ig^+ cells and acidophilic granulocytes (Picchietti *et al.* 2007, 2008, 2009; Salinas *et al.* 2008). Moreover, Picchietti *et al.* (2009) demonstrated local gut immunity of sea bass by an increase in T lymphocytes after supplementation of bioencapsulated *L. delbrueckii* subsp. *delbrueckii*. In addition, an enhanced gut mucosal lysozyme (Kim and Austin 2006b) and phagocytic activity of mucosal leucocytes (Balcázar *et al.* 2006c) by probiotics was also reported. These data suggest that the gut of fish is immunocompetent. Certainly, a strong uptake capacity of bacterial antigens in the gut of the rainbow trout and cyprinid fish has been documented after oral and anal administration, which was subsequently reported to be processed by intraepithelial macrophages (see Hart *et al.* 1988; Nayak 2010). Likewise, particulate antigens (namely whole viruses and bacteria) seem to be taken up by the gut of tilapia and mirror carp (see Hart *et al.* 1988). However, it should be noted that, as in mammals, fish do not have Peyer's patches, secretory IgA and antigen-transporting M cells (= phagocytic cells) in the gut, but hold numerous diffusely structured lymphoid cells, macrophages, granulocytes and mucus IgM representing the gut immune system (see Nayak 2010).

While probiotics are deemed to be beneficial, a question may still arise as to whether or not they exert stress or physiological problems to the host. Recently, Hernandez *et al.* (2009) observed an enhanced tolerance to stress (= significantly higher recovery rates after 1 h of air exposure) of juvenile Porthole livebearer *Poeciliopsis gracilis* fed with *Artemia* nauplii enriched with *L. casei*, when compared with groups fed with just the

non-enriched nauplii. Similarly, the application of probiotics has been recognized to improve tolerance of other fish species to environmental stressors. For example, juvenile Japanese flounder fed with commercial preparations of *B. subtilis*, *L. acidophilus*, *C. butryricum* and *S. cerevisiae* were more resistant to heat shock and air stress, and found with significantly higher plasma lysozyme activity as a measure of stress parameters (Taoka *et al.* 2006a). Likewise, probiotics have been reported to enhance salinity tolerance in tilapia (Taoka *et al.* 2006b). Moreover, there was a lower cortisol (= a hormone directly involved in stress responses that shows higher levels under stress conditions) response in probiotic treated fish during pH-induced stress (Rollo *et al.* 2006) and transportation (Gomes *et al.* 2008). Furthermore, juvenile European sea bass fed with *L. delbrueckii* subsp. *delbrueckii* possessed lower levels of whole-body cortisol compared to controls, which was indicative of better tolerance to rearing conditions by fish treated with probiotics (Carnevali *et al.* 2006). In this study, the levels of serum glucose were statistically similar between the different groups. However, how the probiotics affect the physiology of stress response in fish or lessen the stress stimulus is not clear, but it is believed to be related to improved host health which is directly attributable to probiotics (Rollo *et al.* 2006). A high quantity of total serum protein, albumin and globulin, as recorded in this study, is considered likely to be associated with a stronger innate immune response in fish (Wiegertjes *et al.* 1996), and may be equally linked to the nutritional status and the integrity of the vascular system and liver function (Abdel-Tawwab *et al.* 2008). This argument is supported in this study by the increased survival of the experimental group of rainbow trout after infection with pathogenic vibrios. Certainly, enhanced total protein (Nayak *et al.* 2007; Newaj-Fyzul *et al.* 2007) and higher albumin and globulin levels (Wang *et al.* 2008) in fish have been reported after administering probiotics. The data for serum urea and creatinine showed no statistical difference between groups, and suggest that there were no adverse effects of dietary probiotics on the kidney function of rainbow trout in the current study.

It is relevant to address the potential structural, i.e. anatomical and morpho-physiological changes of the fish digestive tract upon feeding probiotics. Ringø *et al.* (2007) did not observe any changes in the gut histology (i.e. an intact mucosal epithelium) when the foregut of the Atlantic salmon was exposed *in vitro* to *C. divergens* (isolated from the foregut of the Artic charr, *Salvelinus alpinus*) or to Ringer's solution (= control sample). This suggests that non-pathogenic indigenous

bacteria do not affect gut cellular integrity (Ringø *et al.* 2004). Moreover, Sáenz de Rodrigáñez *et al.* (2009) demonstrated that the use of probiotics positively influenced the intestinal functionality of juvenile Senegalese sole (*Solea senegalensis*) with evidence of moderate accumulation of lipids within the enterocytes as compared to big lipid inclusions (= intracellular damage and loss of cytoplasma) in fish fed control diets. The microvilli were also larger and more numerous than the controls. Similarly, Zhang *et al.* (2009) observed that dietary administration of *Halomonas* sp. improved the integrity of the intestinal mucosal layer of shrimp *Fenneropenaeus chinensis*; for example, the intestinal cells or enterocytes were covered by a layer of intestinal mucosa, the arrangement of epithelial cells was more compact and regular in the midgut, and the intestinal mucosal layer was denser in shrimp treated with probiotic than that of the controls.

There is no doubt that probiotics lead to improved health status and enhanced disease resistance of aquacultural species, but there appears to be differences in their activities. In particular, an improper dose and/or feeding duration may lead to unfavorable results (Nikoskelainen *et al.* 2001; Son *et al.* 2009; Merrifield *et al.* 2010c; Nayak 2010). Therefore, age, physiological status and genetic makeup of the fish, rearing conditions, the probiotic feeding duration and dosage, the type and composition (i.e. mono-species or multi-species) of the probiotic, the method of administration and supplementation form, and the interactions of probiotic with the gut microflora could be the determinants influencing the outcome of probiotic treatment (reviewed by Merrifield *et al.* 2010c; Nayak 2010).

In conclusion, dietary supplementation of *Kocuria* SM1 and *Rhodococcus* SM2 demonstrated effectiveness for the control of vibriosis in rainbow trout. Clearly, there is a role for probiotics in fish disease control strategies, and their use may replace some of the inhibitory chemicals currently used in fish farms.

4.2 Conclusions

1. The guts of healthy rainbow trout are suitable locations for the selection of candidate probionts, though only a small proportion may be antagonistic to pathogens.
2. Probiotics SM1 (*Kocuria* sp.) and SM2 (*Rhodococcus* sp.) conferred marked reductions in mortalities of rainbow trout after challenge with *V. anguillarum* and *V. ordalii*, respectively, when compared to the controls. This demonstrates that probiotics are not necessarily specific to single diseases.
3. A two week feeding regime with SM1 dosed at ~10^8 cells g^{-1} feed led to the best protection.
4. An equi-mixture of SM1 and SM2 significantly enhanced fish survival against *V. anguillarum* or *V. ordalii* infections, but the result was not better than the use of single cultures of probiotic.
5. The beneficial effect of dietary SM1 to the defense mechanisms of rainbow trout extends beyond (= up to 4 weeks) the withdrawal period of the feeding regime.
6. The use of cell wall proteins (CWPs) and whole cell proteins (WCPs) of SM1 and SM2 led to significant resistance against *V. anguillarum* infection in rainbow trout. However, extracellular proteins (ECPs) of both probiotics fared less well. This may explain the parts of the cells involved in protection.
7. Probiotics or their subcellular components stimulated the cellular and humoral innate immune parameters in rainbow trout.
8. Improvement of specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) was observed in fish fed with SM2 compared to the control group, but the results were statistically indifferent.
9. SM2 demonstrated slightly better extracellular enzymatic activities compared with SM1, but it is not clear whether the difference led to better growth in fish treated with SM2.
10. Stress or physiological problems in rainbow trout were not observed after administration of dietary probiotics, since the kidney function and the level of serum glucose was similar between groups.
11. The possible mode of action of probiotics is multifactorial, and may include competitive exclusion, nutrition, enzymatic contribution to digestion, and stimulation of innate immunity.

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